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ABSTRACT

This fourth volume of the Science as a Way of Knowing project contains 12 papers given at a conference in 1986 which was cosponsored by a variety of scientific and science education organizations. The presentations include: (1) "Science as a Way of Knowing--Developmental Biology" (John A. Moore); (2) "Embryonic Induction" (Richard M. Eakin, impersonating Hans Spemann); (3) "Understanding Embryonic Development: A Contemporary View" (Eric H. Davidson); (4) "Determinants of Early Amphibian Development" (John C. Gerhart); (5) "Cell Lineages and Determinants of Cell Fate in Development" (J. Richard Whittaker); (6) "Genomic Potential of Differentiated Cells Analyzed by Nuclear Transplantation" (Marie A. DiBerardino); (7) "Cell Adhesion as a Basis of Pattern in Embryonic Development" (Gerald M. Edelman and Warren J. Gallin); (8) "Inheritance of Pattern: Analysis From Phenotype to Gene" (Paul B. Green); (9) "Limb Development and Regeneration" (Susan V. Bryant, David M. Gardiner, and Ken Muneoka); (10) "Developmental Biology and Human Concerns" (E. Peter Volpe); (11) "The Next Big Problem in Developmental Biology" (John Tyler Bonner); and (12) "Films and Videotapes in Developmental Biology" (Gary Anderson and Nathan H. Hart). An index is included. (TW)

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SCIENCE AS A WAY OF KNOWING

An Ongoing Project of the
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SCIENCE AS A WAY OF KNOWING
IV—DEVELOPMENTAL BIOLOGY

Annual Meeting, December 1986, Nashville.

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IV—DEVELOPMENTAL BIOLOGY

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IV

SCIENCE AS A WAY OF KNOWING— DEVELOPMENTAL BIOLOGY

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[1] An impersonation by Richard M. Eakin.

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A grant from the Carnegie Corporation of New York permits us to give single copies of the *Science as a Way of Knowing* publications to those who request them—as long as the supplies last. We are especially anxious to provide them for teachers of introductory biology courses in the universities, colleges, and high schools. There is no charge.

Should you wish a complete copy of *Developmental Biology* (pages 411–734 in this issue), please send your request to: John A. Moore, Department of Biology, University of California, Riverside, CA 92521.

Copies of two previous publications, *Genetics* and *Human Ecology*, are still available. Requests, again, should be sent to Moore.

Multiple copies for classroom use are sold at cost, which is $6.00 each for *Human Biology* and *Genetics*. The cost for *Developmental Biology* will probably be the same. For information write: American Society of Zoologists, 104 Sirius Circle, Thousand Oaks, CA 91360. Telephone number: 805 492-3585.

Some of the Speakers at the *Science as a Way of Knowing—Developmental Biology* Symposium

Left. Richard M. Eakin (University of California, Berkeley) as Hans Spemann. Below. Luncheon for the speakers. Front row, left to right: Paul B. Green (Stanford University), Milton Fingerman (Tulane University), M. Patricia Morse (Northeastern University), William V. Mayer (Biological Sciences Curriculum Study and University of Colorado), Ingrith Deyrup-Olsen (University of Washington), John Tyler Bonner (Princeton University). Rear row, left to right: Leonard Muscatine (University of California, Los Angeles), William R. Dawson (University of Michigan), John C. Gerhart (University of California, Berkeley), E. Peter Volpe (Mercer University School of Medicine), John A. Moore (University of California, Riverside), and Eric H. Davidson (California Institute of Technology).

AMER. ZOOL., 27:415–573 (1987)

Science as a Way of Knowing—Developmental Biology[1]

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Riverside, California 92521

SYNOPSIS. This essay is part of the fourth yearly presentation of an educational project of the American Society of Zoologists. The purpose is to offer suggestions for improving the first-year biology courses in colleges and universities. We emphasize the conceptual framework of the biological sciences, show how scientific information is obtained and validated, and relate science to human concerns. The topic for consideration this year is *Developmental Biology*. This essay gives some of the background information—mainly classical experimental embryology. The speakers in the symposium will deal with more recent discoveries and insights.

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[1] From the Symposium on *Science as a Way of Knowing—Developmental Biology* presented at the Annual Meeting of the *American Society of Zoologists*, 27–30 December 1986, at Nashville, Tennessee.

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INTRODUCTION

Andrew Bard Schmookler (1984, p. 5), although writing about the study of civilization, describes a general problem in the study of biological science:

> In an age of specialized analysis, there is a prejudice against general questions and general answers: the study of the forest is considered best pursued as the study of particular trees. Even as pictures from satellites open our eyes to sweeping vistas, our world view tends to be myopically mired in the magnifying-glass stage. The parts are delineated in excruciating detail, whereas the whole is left for some invisible hand to assemble or is regarded as no more than the sum of its parts.

A common defect in biology courses taught in the colleges and universities is that students are not provided with a satisfying and useful vista of that forest. We tend to forget that learning is most effective when the student starts with the personal world of things and processes experienced and then confronts the unknown world of concept and abstraction.

But that world of concept and abstraction may not be easy for a student to enter. For many of them schooling has consisted of learning facts—true and eternal facts. Those who study cognitive development suggest that the ability to relate phenomena, to explain them symbolically, and to find joy in seeking answers to unsolved problems develops slowly and for many human beings remains always in a stage of incomplete development.

COGNITIVE DEVELOPMENT

There is increasing evidence that the period including high school and the first two years of college sees important changes in cognitive development. Jean Piaget, the Swiss psychologist, suggests that cognitive development is part of biological development (of course neither takes place in the absence of an environment) and that rather definite stages can be recognized. During the elementary school years there is a concrete-operational (or empirico-inductive) mode of problem analysis. The emphasis is on concrete things and empirical evidence. Flavell (1985, p. 98) describes this stage:

> His is an earthbound, concrete, practical-minded sort of problem solving approach, one that persistently fixates on the perceptible and inferable reality right there in front of him. His conceptual approach . . . does, however, hug the ground of detected empirical reality rather closely, and speculations about other possibilities—that is, about other potential, as yet undetected realities—occur only with difficulty and as a last resort. A theorist the elementary school child is not . . . . The realm of abstract possibility is seen as an uncertain and only occasional extension of the safer and surer realm of palpable reality.

During adolescence (11–15 years) a shift is made to a formal-operational (or hypothetico-deductive) level of problem analysis. Here the thinker

> inspects the problem data, *hypothesizes* that such and such a theory or explanation might be the correct one, *deduces* from it that so and so empirical phenomena ought logically to occur or not occur in reality, and then tests her theory by seeing if these predicted phenomena do in fact occur . . . . If you think you have just heard a description of textbook scientific reasoning, you are absolutely right (Flavell, 1985, pp. 98–99).

A college or university course in biology must be based on the students' ability to employ formal-operational, or hypothetico-deductive, reasoning. There are some disturbing indications, however, that students may not have reached this stage by the time they are ready to enter the colleges and universities. Thus Renner *et al.* (1976, p. 96) found that 66 percent of 12th grade students were still in the concrete-operational stage, 15 percent were transitional, and only 19 percent were formal-operational.

Cognitive development must be due in part to the growth of the brain itself and must be influenced strongly by home, friends, activities, and opportunities but there is a strong suspicion that some is the result of modes of instruction in the schools. Rote learning tested by objective examinations does little to stimulate an inquisitive mind. One suspects that much of the inquisitiveness and creativity of the child is dampened by such schooling and by television, both of which are associated with passivity and conformity. There are ample data to show that when very young students are put in situations where they are given encouragement and opportunity to explore and reason, they quickly reach significant levels of achievement in hypothetico-deductive reasoning.

References to cognitive development

Brainerd (1978), *Flavell (1985), Hamilton and Vernon (1976), Inhelder and Piaget (1958, 1964), Piaget (1954, 1977), Piaget and Inhelder (1969), Renner *et al.* (1976), Rosen (1977), and Smith (1982).

In the references just given the * indicates a title that provides an excellent introduction to the topic being considered. This plan will be followed throughout this essay. The references given are usually those available to me in our campus library but in some cases I have included titles not seen but suspected to be useful.

A SUGGESTED APPROACH TO TEACHING

In any event, many of the students who come to the colleges and universities have had little or no experience with mature conceptual thought and the inquiry approach to learning. Thus it becomes all the more difficult to teach biology, or any science, in the way we suggest. The difficulty in doing so, however, is a measure of prior inadequacies that we must seek to remedy.

The previous volumes of the *Science as a Way of Knowing* series have discussed in some detail our goals and procedures (for example see *Science as a Way of Knowing—I. Evolutionary biology* 1984, pp. 469–476, 524–525; *Science as a Way of Knowing—II. Human ecology* 1985, pp. 486–489; *Science as a Way of Knowing—III. Genetics* 1986, pp. 4–7, 153–154). *Hereafter when reference is made to these earlier publications of this project they will be indicated only by number and page, i.e.,* II, pp. 379–381, etc.

There are some things about the biological sciences that every educated person should know, *i.e.*, organizing ideas that help us to understand and enjoy the natural world. For example, one should be able to look at the vast diversity of life and understand its origin through evolution over vast stretches of time. Knowledge of the structure and function of the human body is not only important in itself but also in maintaining health. Today it is essential to understand the interrelations of all living organisms and the cyclic changes of substance and energy that occur in the environment. Green plants and other living creatures, and their activities, are our life support system, yet in many ways and in many places we are using and abusing the environment beyond its ability to sustain itself—and hence, us. In the eternal quest for resources, all organisms including human beings and their food crops, may become the prey of other organisms and our efforts at control may introduce second-order problems.

As science improves its ability to predict and control natural events, it becomes ever more important that people understand the nature of the scientific process, its strengths and its limitations, and that the importance of science lies in its ability to help us understand and control natural processes. That understanding will provide us with information necessary to reach humane decisions but not to specify what the decisions should be. It is here that science should join ethics and morals in making a better world.

The need for biological knowledge does not end with graduation from the university, and for that reason students must acquire a conceptual framework that will allow the facts of biology to be seen as part of an organized whole. Such a framework becomes a powerful mnemonic device and will provide understanding of new biological facts as they are encountered throughout life.

The goal toward which the *Science as a Way of Knowing* project strives is

> primarily concerned [not] with the history of discovery, but rather with those fundamental conceptions which are ageless and persist, however much they may be altered, extended, or transformed by the discovery of new facts (E. S. Russell, 1930, p. 25).

It must be emphasized, however, that a conceptual framework is something that is worked toward, not started with. Learning is made easier if a variety of biological phenomena are first selected, then studied, and finally united in conceptual schemes that can be tested by the usual procedures of logic and science. That is, a question about some natural phenomenon is asked, a possible answer is framed as a hypothesis, deductions are made and these are tested by observation and experiment. Thus one may reach a level of understanding that, for the time, can be said to be true beyond all doubt. Once a concept has been established as true beyond all reasonable doubt, it serves to organize observations and information acquired subsequently.

This essay will attempt to provide such a conceptual framework for developmental biology. It will be concerned mainly with the sorts of questions that have been asked about development and the scientific procedures employed to answer them. The emphasis will be not on what scientists are working on today but what they have found out. This seems appropriate if the goal is to establish the conceptual framework of the field. When dealing with the past, one knows not only the questions but the procedures for answering them and the answers themselves. The emphasis will be on science as a way of knowing.

The intent is not to move back to some Golden Age of Embryology but to better understand the major concepts of developmental biology. These are relatively secure and they will provide a framework for which current research is attempting to provide a molecular basis.

But, of course, there must be a balance. Research in progress portrays science as a way of trying to find out. This can be a stimulating approach to some while it is threatening to others—because of the student's insecurity of not knowing *the* answer when it is yet unknown but the student assumes it to be necessary by exam time. Thus much of the old and a little of the new may be a balance well suited for first-year students.

This essay is not a history of science. Its intent is to marshal the data that have led to our understanding of developmental phenomena. Since the first questions tend to have been asked at an earlier time than later, the essay will reflect history rather than be it. The discussion of ideas and data is not in strict historical sequence but when it has deviated it is to present a better analysis of a problem. For example, the Spemann-Mangold dorsal lip experiment, although first in time, comes as the climax of work leading to the organizer.

I have selected some key individuals and their discoveries and neglected even more key persons and their accomplishments. Some readers may object that E. B. Wilson will come in for more attention than he may deserve but he was both so outstanding and so quotable that I do not apologize for telling so much about what he did. In many instances I have included long quotations in the belief that original statements will be more valuable than my interpretations.

It is important, I believe, that ideas be associated with individuals. For many students ideas in science come across as a rhetoric of conclusions with no notion of person, place, or time. This is not only regrettable but it makes it ever so much more difficult for a student to imagine what role he or she might play in science. After all, science is a human enterprise—so shouldn't we teach it as such?

PROBLEMS AND PROMISES

The field of developmental biology has long had an unsatisfying element—a lack of conceptual coherence. Its problems are central to biology—how the new individual is deciphered from the universal code—yet their conceptualization remains elusive. Horder (in Horder *et al.*, 1986, p. ix) refers to

> a sense of puzzlement concerning the present state of the discipline of embryology, where, despite all our massive knowledge about embryos at the descriptive level and their basis in molecular and cell biology, the nature of embryological events is generally viewed as mysterious and unsolved . . . . Embryology represents a distinct and significant domain among the biological phenomena, as open to satisfying explanation as any other, and . . . it is a subject which, since embryogenesis has been a precondition for the very existence of living forms throughout evolution, [it] ought to occupy a key position in biology, many areas of which stand to benefit if it were better understood.

In spite of all the dashed hopes, developmental biology may be about to come into its own.

FIRST QUESTIONS—FIRST PRINCIPLES

In contrast with so many other modes of inquiry, science advances by studying what is not known rather than by studying what is already known or assumed to be known. For the working scientist, answers may be interesting and satisfying but their true importance is as a basis for asking new questions—science is process, not position.

Thus the beginning of an inquiry in science is the posing of a question about some puzzling phenomenon of nature. This is not a trivial exercise. Important answers will be obtained only if the question relates to some fundamental aspect of the natural phenomenon, and only if there exist practical means of searching for an answer. Many important questions about nature remained unanswered for millennia and many remain so today mainly because there were or are no methods for initiating the inquiry. Some of the early questions about disease, for example, could not be answered until microscopes had been invented and the previously invisible pathogens could be observed. In fact, satisfying answers to many basic biological questions were unobtainable until the invisible world of life could be entered with the techniques of both microscopy and biochemistry.

And so it has been with developmental biology or, as it was better known over its long history, embryology. It may come as a surprise, therefore, to find that Aristotle (384–322 B.C.), that Greek of universal intelligence, not only established the discipline of embryology but posed the major questions that have lasted to today. But then, according to E. S. Russell (1930, p. 2) this may not be surprising at all.

> In spite of the vast accumulation of detailed knowledge, which is, in some quarters, supposed by itself to constitute science, there is much less difference than one would expect between the fundamental hypotheses or modes of explanation adopted, say, by the Greeks and those in vogue at the present day. This is because there are—apparently—only one or two possible ways of interpreting development open to the human intelligence, and these few alternative methods tend to recur again and again throughout the whole history of biological science. One is accordingly forced to the conclusion that on its constructive or theoretical side biology (and perhaps the other sciences as well) is by no means a simple transcript of fact, but in large measure a construction of the mind, a conceptual edifice, the lines and plans of which may vary according to the type of mind of its architect.

These basic questions may not seem difficult to students since we usually tell them what they are. Their approach to embryology, however, will be more instructive if *they* are asked to suggest what the questions might be and how answers might be sought. This can prove a valuable exercise, especially if those members of the class who have never studied biology are asked first "What are the questions one would like to know about development?" Possibly your students will recapitulate Aristotle and, as Russell suggests, ask the same questions.

This is the problem of development as Russell (p. 1) saw it in 1930:

> The general problem of development is without question one of the most difficult and intriguing in the whole field of

knowledge. That from a minute germ of relatively simple structure there should be gradually built up, by a series of processes beautifully co-ordinated in space and time, the complex organization of the adult is a fact that has never ceased to excite the wonder of mankind. It has provided a constant challenge to the intellect of man, and many and various have been the theories invented to explain it. It ranks as one of the major problems of biology.

But what are the questions that are formulated in such a manner that they can be answered? It may be difficult for your students to suggest good questions but the very fact that they try is most important. Students are rarely asked about matters so basic as this but, surely, a science course can be expected to stimulate their latent heuristic minds. So after listing some questions posed by naive persons today it will be interesting to see how a person, initially even more naive and living two and a half millennia ago, tackled the problems.

THE PERIPATETIC STAGIRITE

The extant biological works of Aristotle consist of *Historia Animalium*, which is a general biology of animals; *De Partibus Animalium*, a comparative physiology and anatomy of animals (Sarton, 1952, p. 532, call this the first animal physiology in any language); *De Motu Animalium*, dealing with movement and some aspects of psychology and metaphysics; *De Incessu Animalium*, also concerned with locomotion; *De Anima*, considering the vital principle of living things; *Parva Naturalia*, mainly psychology; and *De Generatione Animalium*, Aristotle's treatment of developmental biology.

Scholars are reasonably sure that existing forms of these works are relatively accurate. During the Renaissance, when Aristotle's works became known in Western Europe from Arabic editions, it was suspected that translations from Greek to Arabic to Latin might have introduced errors. Subsequently manuscripts in Greek were discovered and these are assumed to be closer to the originals. To be sure some are suspected of containing not only errors made when the manuscripts were copied but also there is sometimes evidence of attempted independent creativity on the copyist's part. When several different manuscripts of the same work are available, however, these errors and insertions can usually be detected and expunged. None of the extant manuscripts are very old. For example, the oldest of the nine most important Greek manuscripts of *Historia Animalium* dates from the 12th or 13th century and the rest are from the 13th to the 16th century. To keep this in perspective: the interval from Aristotle to the 12th or 13th century is roughly the same as the interval from the end of the Roman Empire in the West (476 A.D.) to the present.

HISTORIA ANIMALIUM

Historia Animalium is the earliest known animal biology text and its scope and originality are astonishing. Aristotle knew a very great deal about a very large number of organisms. He was interested in their structure, breeding habits, reproduction, behavior, ecology, distribution, and relationships.

So far as developmental biology is concerned, *Historia Animalium* contains a large amount of factual material that is used for the more theoretical considerations of *De Generatione Animalium*. The "basic facts of life" were known, of course, in Aristotle's time and he described what was believed and suspected of reproduction in human beings and many other animals. Reproduction and development were so basic to understanding the biology of organisms that Aristotle used viviparity and oviparity as important characteristics in classifying organisms (*HA* 489^a, 35ff. It is customary to identify the sections and sentences in Aristotle's works in this manner, which refers to the standard edition of Bekker (1831–1871); *HA* is *Historia Animalium*).

It was generally accepted that development began after "something," assumed to be secretions, from the male parent and the female parent became associated. In the case of human beings the male secretion was semen and the female's contribution was assumed to be something like menstrual blood. No one living at that time, or for the subsequent two millennia, had any accurate notion of sperm or ova. Aristotle knew that many animals produced visible eggs and that from these the young slowly developed. But since other species did not seem to have eggs, there must be "something" more basic.

> What we term an egg is a certain product of conception from which the animal will develop . . . the developing embryo comes from only part of the egg and the rest serves as its food (*HA* 489^b, 6).

Aristotle was familiar with the early embryos of numerous animals, and his most complete description is of the developing chick (*HA* 561^a, 3–562^a, 21). When this is read by a biologist today, it sounds so familiar that one tends to forget the tremendous intellectual steps that Aristotle took when he wrote: "Development from the egg proceeds in an identical manner in all birds." This implies that Aristotle was familiar with development in at least a few other species and, assuming a basic uniformity of natural phenomena, felt secure in extending the conclusions based on a few species to all species of birds.

His belief that at a fundamental level nature is not capricious is a necessary working premise for all scientists. Today we feel confident that, for all intents and purposes, the genetic code is universal, yet that confident feeling is based on acceptable data for no more than a trivial fraction of one percent of all species.

Of great importance was Aristotle's use of data from as many different species as he could obtain. This is so basic to biology today that we accept it as the obvious thing to do. When the comparative method is used, one sees variations in the phenomenon being studied—with some species giving a glimpse into one part of the process and another species giving a different glimpse. Each species, in a sense, is an experiment and, when all of the observations have been made, there is a better chance of understanding the fundamentals of the phenomenon. In the early days of cytology and genetics this procedure was basic for establishing the concepts of those fields (for example, III, pp. 669–670). Observations on the kidney of the goosefish were basic for the discoveries of how the mammalian kidney functions. Time and time again it has been found that if you cannot obtain an answer from one species, try another and you may succeed.

We must note that Aristotle was busying himself with an essentially "useless" task. His biology was not making people either richer, or better, or producing better crops, or helping to fight disease. Aristotle was, instead, providing materials for the inquisitive mind. This was "pure science," that is, science for its own sake. For many centuries attempts such as his to understand the natural world were followed by only a few, often lonely, individuals; and for the most part there were to be few practical fruits of their studies until the Renaissance.

And in contrast with the way many people reasoned then and now, Aristotle tried to base his search for the "hows" and "whys" on the "whats."

Historia Animalium has a very large amount of data on the development of the chick. Aristotle reported that the first visible indications of the embryo came after three days but earlier in small species of birds and later in large species. At this time the heart appears as a tiny red spot, it pulsates, and what we now call the vitelline veins are seen to be carrying blood. A little later the body differentiates and the head, with very large eyes, can be made out.

All these observations were made without a microscope, so he was working at the limits of the unaided eye. His description of the much larger embryo at 10 days is far more complete. The head and the eyes are relatively large, and the main internal organs are visible. He provides a fairly accurate description of the embryonic membranes—those structures so baffling to our students in embryology courses today. He even dissected the eye of the 10-day chick.

Some later observers belabored Aristotle for saying that the heart is the first structure to develop. He did not quite say that but said "Blood is developed first of all in the heart of animals before the body is differentiated as a whole." That is a perfectly reasonable statement if made when magnification was impossible and Lillie's *The Development of the Chick* unavailable for reference. One sees blood because it is red while the rest of the embryo is not only tiny but also mostly colorless. One must admit that Aristotle did not know all we know today but one might hope that he would be celebrated for his enormous accomplishments in bridging the gap from no science to science. Some detractors would not be satisfied, I suspect, unless Aristotle had come down from the heavens, landed on the Acropolis and said:

Δνα → ρνα → προτειν

Aristotle devoted Chapter VII of *Historia Animalium* mainly to human reproduction and development and he describes a human embryo of forty days, when it was as big as one of the larger ants. His observations appear to have been made on an aborted embryo.

There are many other observations in *Historia Animalium* dealing with embryology. He notes, for example, that in a general way development in birds and fishes is the same (*HA* 564^b, 30) and that development is the same in fishes that are oviparous internally and oviparous externally (*HA* 567^b, 27). Aristotle was discovering the natural order behind the putative chaos.

It is clear that Aristotle added the observations of others to his own (Preus, 1975, pp. 21–47). In so doing he suffered the fate of Darwin, who in *The Variation of Animals and Plants under Domestication* included erroneous observations, such as that on Lord Morton's mare, that rendered accurate conclusions impossible (III, pp. 602, 603). For example Aristotle quoted reports that the sorts of water drunk by rams could determine the hair color of the lambs they sired. If the water was from Assyritis the lambs were black; they were also black if the water was from one river in Antandria, but white if from another; consumption of water from the Scamander River caused the lambs to be yellow (519^a, 10–20).

If one accepts these observations, which Aristotle probably obtained from others, one must conclude that inheritance and development are extremely labile and easily influenced by external conditions. Like need not beget like all of the time.

DE GENERATIONE ANIMALIUM

There are similar problems in *De Generatione Animalium* of basing a theory on incorrect observations. Although many animals, especially those with blood, produce young as a result of copulation, the young of some develop from decaying matter or feces (*GA* 715^a, 25; 715^b, 5). Thus, genetic continuity cannot be true for all species.

De Generatione Animalium contains many observations, some repeated from *Historia Animalium*, about the nature of semen and how the embryo is formed from it. Observations on very many species are offered and it is clear that Aristotle had more first-hand experience with a variety of developmental patterns than most embryologists today. He organized the data to answer specific questions and to develop general principles.

First he sought to establish what it is that parents transmit to offspring. He accepted that it must be substance and can be called "semen" in both fathers and mothers. The first question considered, which was not posed initially by Aristotle, was the relation of the structure of the body to what was in the semen (721^b). One prevailing view was that every part of the adult body contributes some specific material to the semen—a notion that, millennia later, was to be known as the Theory of Pangenesis. Some of the observations and arguments in support of pangenesis are given but Aristotle thought them not convincing. One of his arguments, paraphrased, is as follows (722^a, 35ff.): If flesh and bones are constructed out of fire and similar substances, the semen would have to be drawn from the element fire in flesh and bones of all sorts. Reduced to this elemental state, the elements in semen would not be one sort of fire from flesh or another sort of fire from bones. Therefore, "Blood is formed out of something that is not blood" (723^a, 5).

Aristotle is proposing that something more fundamental than a specific structure must be transmitted in semen. This is inevitable since there are very few elements, probably just four of which fire is one, and how would the elements "know" they were to form specific sorts of flesh and bones and not something else composed mainly of the element fire?

Another of his arguments against pangenesis is that if all parts of the body of the male parent and all parts of the female parent produce something that is transmitted, then the result should be two embryonic bodies, not just one. Or in those cases where there are many offspring how can it be that the specific determinants for all of the body structures are packaged so that every offspring gets that entire package (729^{a}, 5ff.)?

Aristotle concludes that either the semen does not come from all parts of the body or, if it does, some additional mechanism must be responsible such as one attributed to Empedocles: each parent contributes only part of what is required to form a complete body and sexual intercourse is needed so their semens can join to form the entire offspring (722^{b}, 10ff.).

Thus one can read into Aristotle the notion that parents transmit not structures to their offspring but "information" to construct those structures in the course of development.

Aristotle suspected that the contributions of male and female parent are quite different. The semen of the female was thought to be menstrual fluid (729^{a}, 26) and it differed in a fundamental way from male semen in supplying the substance (727^{b}, 32) for the embryo whereas male semen (728^{a}, 30) supplies the form and principle of movement (which can probably best be thought of as meaning "animal life"). The action of male semen on the female secretion was thought to be analogous to the action of rennet upon milk. Rennet "sets" the homogeneous milk just as male semen "sets" the menstrual fluid (739^{b}, 20).

When it comes to the formation of the embryo itself, the analytical mind of Aristotle reasoned that it must be formed out of something, by something, into something (733^{b}, 25). The "out of something" is the life-giving material in the semen of the male plus the material substance supplied by the female. The "by something" is assumed to be carried in the semens of both parents. When it turns "into something," *i.e.*, develops, Aristotle considers two possibilities. Some philosophers held that all parts of the embryo's body form at the same time. Aristotle refuted this hypothesis by observation—the heart in the chick embryo appears before the lungs. One cannot deny the validity of this observation, says Aristotle, by suggesting that the lungs are too small to see because, in fact, they are larger than the heart and hence should be visible first. New things appear in the course of development.

Thus, in this longest of any debate in embryology, preformation *vs.* epigenesis, Aristotle comes down on the side of epigenesis.

He makes clear his belief that the semen transmits only the potential for the embryo's structures, not the actual structures themselves (737^{a}, 20). The potential is in the female's contribution to which the male provides the mechanism for potential to become actual (740^{b}, 20ff.). E. S. Russell (1930, p. 17) recognizes this as a basic point and says,

> [Aristotle's] fundamental idea, that development is the functional actualization of a functional potentiality, is a profound one and gets down to the root of the matter.

Aristotle has so much to say about so many things that it is easy to read many modern ideas into his statements. One might be tempted, for example, to see in his remarks about some structures being formed first that are necessary for later developments (742^{a} and 742^{b}) an anticipation of organizer theory. There are many similar statements.

But Aristotle obviously was not always correct in his biology. He was convinced, for example, that spontaneous generation was the rule for some creatures (762^{a}, 763^{a}). This belief was based on many observations of the apparent generation of some insects from decaying matter and the appearance of marine invertebrates on pots and other objects placed in the sea. It took a large amount of careful observation and experimentation by many naturalists, from Redi (1626–1698?) to Pasteur (1822–1895), to bell that cat.

ARISTOTLE'S ACCOMPLISHMENTS

Joseph Needham, the famous embryologist and even more famous historian of Chinese science and technology, credits Aristotle with extraordinary accomplishments (1959, p. 42).

> [Aristotle] stood at the very entrance into an entirely unworked field of knowledge; he had only to examine, as it were, every animal that he could find, and set down the results of his work, for nobody had ever done it before . . . . The extraordinary thing is that building on nothing but the scraps of speculation that had been made by the Ionian philosophers, and on the exiguous data of the Hippocratic school, Aristotle should have produced, apparently without effort, a text-book of embryology of essentially the same type as Graham Kerr's or Balfour's . . . . The depth of Aristotle's insight into the generation of animals has not been surpassed by any subsequent embryologist, and, considering the width of his other interests, cannot have been equalled.

Aristotle sought to understand by first observing; realizing that general concepts might emerge from the study of the same phenomenon in a variety of species; recognizing the fundamental similarity of development in fish, bird, and mammal; arguing for a physical basis of inheritance; providing argument and observation to support epigenesis; and in suspecting that problems of development and regeneration are similar.

And there are a host of minor observations of great interest. For example, one reads with incredulity his realization that nails, hair, and horns all form from the skin (745^a, 20). But as Dante was to say in the *Divine Comedy* (*Inferno*, Canto IV), Aristotle was the "Master of them that know."

One of the most basic contributions that Aristotle made to the field of developmental biology was that he got it started. He collected all the data he could, in a true Baconian fashion, and tried to bring order to the seemingly random phenomena. Considering the time and the newness of such concerns, he did remarkably well. His scientific methodology was deficient only in lacking the widespread use of controlled experimentation.

D'Arcy Thompson (1922, p. 144) praised him for yet another accomplishment:

> He was the first of Greek philosophers and gentlemen to see that all these things were good to know and worthy to be told. This was a great discovery.

Possibly his greatest overall contribution to biology was his firm belief in naturalistic interpretations. This comes through clearly in his attempts to understand the generation of bees. He concluded (760^b, 29ff.):

> This, then appears to be what can be said about the generation of bees—at least as far as theory and what appear to be the facts can take us. But the facts have not been firmly established. If at any future time they are ascertained, one must rely on observations rather than theories—and on theories only if they agree with the facts.

That's a splendid statement. Unfortunately that sound advice was rarely heeded by Aristotle's followers. Arthur Platt, in his translation of *De Generatione Animalium* (footnote 760^b), comments as follows:

> It should have been kept in mind by those bastard Aristotelians who at the revival of learning refused to accept observed facts because they were supposed to contradict Aristotle's statements.

References to Aristotle

The primary sources are Aristotle's *Historia Animalium* and *De Generatione Animalium.* The Loeb Classical Library and Oxford University Press editions, and Balme have useful comments by the translators.

Other sources are Adelmann (1942,

*1966), Balss (1936), Cole (1930), Downey (1962), Düring (1966), Farrington (1949), Grene (1963), Jaeger (1948), Locy (1925), Lones (1912), Magner (1979), Morsink (1982), *Needham (1959), Oppenheimer (1955, 1971*a*), Owen *et al.* (1970), Peters (1968), Preus (1970, 1975, 1977), Randall (1960), Ross (1930), E. S. Russell (1930), Sarton (1952), Singer (1922, 1960), D'Arcy Thompson (1922, 1940), and Woodbridge (1965).

THE DAWN OF NATURALISTIC THOUGHT

One of the most astonishing events in intellectual history is the sudden appearance, seemingly *de novo,* of naturalistic thought—so dominant in the science of Aristotle. This is the procedure of basing explanations of natural phenomenon on the things and processes of nature. For example, when ascertainable and specific meteorological conditions prevail, liquid water is precipitated from clouds as rain. This is in marked contrast to supernatural or mythical explanations, which assume that some god or intangible force is the cause, such as rain is the tears of weeping gods. W. K. C. Guthrie (1962, p. 40) credits Aristotle with contrasting these polar modes of thought:

> It is to Aristotle in the first place that we owe the distinction between those who described the world in terms of myth and the supernatural, and those who first attempted to account for it by natural causes. The former he called *theologi,* the latter *physici* or *physiologi,* and he ascribes the beginning of the new, 'physical' outlook to Thales and his successors at Miletus, hailing Thales himself as 'first founder of this kind of philosophy'.

"This kind of philosophy" has been fundamental to the advance of science.

Miletus, a seaport on the Ionian coast (now Turkey), was settled by Greeks about 1000 B.C. It was the home of three philosophers who, in the absence of earlier evidence, are the first we know who systematically used naturalistic thought to explain natural phenomena. Thales (*ca.* 625–547 B.C.), the first, was followed by his pupil Anaximander (*ca.* 611–547 B.C.) and later by Anaximenes (*ca.* 585–528 B.C.). Among other problems these Milesians were concerned with the basic materials of which all physical objects are composed. Thales thought the elemental substance was water, Anaximenes thought it was air, and Anaximander assumed some unknown and even more basic substance.

Aristotle (*Metaphysics,* 983^b, 20ff.) offers the following suggestion for the origin of Thales' view:

> Thales . . . says the principle is water (for which reason he declared that the earth rests on water), getting the notion perhaps from seeing that the nutriment of all things is moist, and that heat itself is generated from the moist and kept alive by it (and that-from-which-they-come-to-be is the principle of all things). He got his notion from this fact, and from the fact that the seeds of all things have a moist nature, and that water is the origin of the nature of moist things.

Thus such elemental stuff as water could be modified as plants, animals, mountains, soil, clouds, etc. Human beings consume water, air, plants, and animals and convert them into human substance and, upon death, all change back to water once again. Hence, it was not too far fetched to suspect that there was a common building block for all matter. This search for elemental particles, of which all substances are composed, has concerned philosophers and later scientists (when the two groups became different after the Middle Ages) until the present. It remained for the English scientist, John Dalton (1766–1844), to provide acceptable evidence for atoms, predictions for which go back to ancient times. In our century, the "indivisible" atoms have dissolved into a hierarchy of subatomic particles.

The specific hypotheses of the Milesians were of little value; it was their approach that was so novel and so important. Many things suggested to Thales

> that *if there is any one thing at the basis of all nature,* that thing must be water. If

> there is any one thing! This supposition, that is to say, the asking as it were of this question, constitutes Thales' claim to immortality. The fact that he made a guess at the answer, and a pretty good guess at that, is of minor importance. If he had championed the cause of treacle as the sole "element" he would still have been rightly honoured as the father of speculative science. True, others before him (such as Homer and Hesoid) had sketched the origin of the world from one substance, but they were not content to deal with *verae causae*, that is with things whose existence can be verified by observation. To attempt to explain the origin and process of the world by having recourse to gods and spirits endowed with special powers, is merely to beg the question, since the existence of such beings can never be proved (nor of course disproved) by the means wherewith we know that world. In a word, it was Thales who first attempted to explain the variety of nature as the modifications of something *in nature* (Wightman, 1951, pp. 10–11).

The Milesians were asking fundamental questions and proposing *naturalistic* hypotheses in contrast to all others who invoked supernatural forces—the earth being formed from the body of the goddess Tiamat, for example.

One might have imagined that such an important philosophical shift would be based on a substantial amount of original written material. In fact, there is none. What is known about Thales is based on brief mention by Aristotle (*Metaphysics* 983^b, 20ff.; 984^a, 2) and a few other ancient writers. Our evaluation of the Milesians, therefore, is based mainly on the opinion of Aristotle—*he* thought they had made an intellectual breakthrough and there is no reason not to accept his conclusion.

The Frankforts (1977, p. 376) offer this paean:

> The Ionian philosophers gave their attention to the problem of origins; but for them it assumed an entirely new character. The origin . . . which they sought was not understood in the terms of myth. They did not describe an ancestral divinity or a progenitor. They did not even look for an "origin" in the sense of an initial condition which was superseded by subsequent states of being. The Ionians asked for an immanent and *lasting* ground of existence . . . .
>
> This change of viewpoint is breath-taking. It transfers the problem of man in nature from the realm of faith and poetic intuition to the intellectual sphere. A critical appraisal of each theory, and hence a continuous inquiry into the nature of reality, became possible. A cosmogonic myth is beyond discussion. It describes a sequence of sacred events, which one can either accept or reject. But no cosmogony can become part of a progressive and cumulative increase of knowledge . . . . Myth claims recognition by the faithful, not justification before the critical. But a sustaining principle or first cause must be comprehensible, even if it was discovered in a flash of insight. It does not pose the alternative of acceptance or rejection. It may be analyzed, modified, or corrected. In short, it is subject to intellectual judgment.

And for Guthrie (1962, p. 70),

> the perennial fascination exercised by the Milesians lies in just this, that their ideas form a bridge between the two worlds of myth and reason.

Your students might find it interesting to estimate the relative frequencies with which they use various sorts of reasoning in making everyday decisions. They may discover that naturalistic thought may not be the prevailing mode even in this Age of Science.

References to Ionian philosophy

Baldry (1932), Cherniss (1951), Cornford (1952, 1957), Dicks (1959), Farrington (1949), Frankfort *et al.* (1977), *Guthrie (1962), W. T. Jones (1952), Longrigg (1976), Magner (1979), Neugebauer (1957), O'Connor (1964), B. Russell (1945),

Sarton (1952), Taton (1963), Waerden (1961), and Wightman (1951).

GALEN

One might have anticipated that the combination of those "right-thinking" Ionians and that omnivorous observer and speculator about nature, Aristotle, would have begun a vigorous investigation of development, as well as other biological problems. Not at all. A peak was reached with Aristotle and, thereafter, there was a decline of interest and accomplishment for roughly two millennia.

The next major figure who wrote on embryological matters, five centuries after Aristotle, was the Greek physician Galen (*ca.* 130–200 A.D.). He was interested mainly in human anatomy and physiology but he did have a few things to say about development (Galen, 1916, book I, chapters 5–7, book II, chapter 3, and book III, chapter 3; Galen, 1968, books 14 and 15; see also Adelmann, 1942, *1966; Kudlein and Wilson, 1972 and Needham, *1959). He added little to Aristotle's work. The following quotation gives the flavor of his views, which were important since Galen's work was known in Western Europe during the Dark Ages when Aristotle's biology was not:

> Genesis [=embryogeny], however, is not a simple activity of Nature, but is composed of alteration [=histogenesis] and of shaping [=organogenesis]. That is to say, in order that bone, nerve, veins, and all other tissues may come into existence, the *underlying substance* from which the animal springs must be *altered.* In order that the substance so altered may acquire its appropriate shape and position, its cavities, outgrowths, attachments, and so forth, it has to undergo a *shaping* or formative process. One would be justified in calling this substance which undergoes alteration the *material* of the animal, just as wood is the material of a ship, and wax of an image (1916, p. 19).

Looking back, with the knowledge of what was to come, we can say that Galen was defining the fundamental problem of development—differentiation. The formative material must be "altered" since the early embryo, which is to become that adult, lacks the tissues and organs characteristic of the adult. The conversion itself would involve a variety of morphogenetic movements. Thus, novelty would appear in the course of development—Galen was an epigeneticist.

He was also the end for centuries. "The death of Galen in 200 A.D. marks the end of progress in embryological learning for over thirteen centuries" (Adelmann, 1942, p. 45). That length of time is really beyond comprehension. The American Revolution seems remote to most of us, yet those thirteen centuries were more than six times the interval between our national birth and today.

THE MIDDLE AGES

There are many possible reasons for those dark centuries. The political and social stability sustained by the Roman Empire was swept aside by degeneration from within and invasion from without. The rise of Christianity and the establishment of the church as the only effective institution in the West changed the topics for serious thought. The problems of the natural world were replaced by those of the supernatural world. The ability to read and write became rare skills. To be sure there was very little to read apart from theology. What education there was consisted mainly of instructions for those seeking a career of service to the Mother Church. Those with interests in science were rare, as they always had been, and insufficient to form that critical mass which is essential for sustained scientific progress. There were no universities where science was taught, no scientific academies, and few libraries. Essentially no Greek science, except for Galen, was available in the West.

But even if these constraints of the Middle Ages had not existed, *what* was one to do in order to extend Aristotle's and Galen's analysis? The answer is far from obvious. The major questions they had raised were not really approachable until the 19th century when it first became possible to work at the cellular level. One could continue to observe the gross features of development in any embryos that were available. Basic processes and causal relations could not be studied.

It is most unlikely that fascination with the mystery of development, especially human development, ever ceased.

> We may assume that every thinking man has asked himself some questions with regard to the formation and development of embryos, for such questions are continually forced upon him by life itself (Sarton, 1931, p. 315).

Needham (1959, p. 65) reports that "Cleopatra, the Ptolemaic queen, had investigated the process of development by the dissection of slaves at known intervals of time from conception, following the precepts of Hippocrates with regard to hen's eggs." Kottek (1981) adds the following to this report, quoting ancient sources:

> It happened that Cleopatra, the Queen of Alexandria, presented to the physicians some of her maids who had been condemned to death and they were dissected. It was found that the male embryo is complete after forty-one days and the female embryo after eighty-one days.

Other versions of the account differ in maintaining that there are no differences in the times males and females are "complete."

There were some isolated observations on embryos during the Middle Ages and early Renaissance. The developing hen's egg was the usual object of study. By the time of Albertus Magnus (1193?–1280), the works of Aristotle were becoming available and Albertus was a close student of the Master's works. He described the development of the chick, but seemingly his information came only from the Master, not an opened egg. That was standard procedure for the Middle Ages.

SCHOLASTICISM

The Scholastic Method for arriving at truth has been much maligned by later scholars. A debased variation of it remains an important pattern of thought for many people to this day. The method consists basically of accepting the opinions of others rather than data personally obtained by observation and experiment. Since the opinions of others might differ, a formal way of seeking "truth" became common: proposition, opposition, and resolution. That is, the question was raised and the supporting answers of accepted authorities were listed. Then the opposing answers were listed and, finally, an attempt was made to resolve the differences and reach some acceptable conclusion. This Scholastic Method was eminently suited for those whose disputations were on theological subjects. In fact, it is hard to think of any other way to decide such questions, short of resorting to violence.

Scholasticism was a unification of theology and philosophy with the central goal of proving the existence of God. It would have been of little importance for science had it not been, for centuries, the dominant mode of thought of intellectuals—the group from which those with an interest in science would have been expected to emerge.

Truth existed in the mind of God and it was the task of mortals to fathom what that truth might be. The procedure was logical reasoning based on scripture, church dogma, and the opinions of revered philosophers. Thus, in the last analysis, all data was derived from revelation and right-thinking people. Faith came first, understanding later.

A notable exponent of scholasticism was Peter Abelard (1079–1142) who, however, exposed the fundamental weakness of the approach. In his famous book *Sic et Non* he lined up the "Yes" and "No" opinions about the same question and showed that equally respected sources could hold diametrically opposed points of view. He had real problems with the Church on that score but even more problems of another sort. This is the Abelard who had an affair with the beautiful and loving Heloise. Her father felt strongly about that and had Abelard castrated to cool his ardor. It did.

Scholasticism precluded science. Even those who were interested in science looked to Aristotle for the answers, not to nature herself—as we have already observed for Albertus Magnus. This was a far cry from those naturalistic Greeks who probed nature with mind to obtain understanding in contrast to those who sought understanding by probing mind with mind.

This point of view may be difficult for us to comprehend today but possibly this appraisal by Brehaut (1912, pp. 67–68) of Isidore of Seville (the author of the most extensively used encyclopedia of the Middle Ages) may help:

> The view held in the dark ages of the natural and the supernatural and of their relative proportions in the outlook on life, was precisely the reverse of that held by intelligent men in modern times. For us the material universe has taken on the aspect of order; within its limits phenomena seem to follow definite modes of behavior, upon the evidence of which a body of scientific knowledge has been built up. Indeed at times in certain branches of science there has been danger of a dogmatism akin to, if the reverse of, that which prevailed in medieval times with reference to the supernatural. On the other hand, the certainty that once existed in regard to the supernatural world has faded away; no means of investigating it that commands confidence has been devised, and any idea held in regard to it is believed to be void of truth if inconsistent with the conclusions reached by science. In all these respects the attitude of Isidore and his time is exactly opposite to ours. To him the supernatural world was the demonstrable and ordered one. Its phenomena, or what were supposed to be such, were accepted as valid, while no importance was attached to evidence offered by the senses as to the material. It may even be said that the supernatural universe bulked far larger in the mind of the medieval thinker than does the natural in that of the modern, and it was fortified by an immeasurably stronger and more uncritical dogmatism.

Isidore of Seville was a man of extraordinary intellectual powers yet he was molded by his time—as we are by ours. Had he been alive today, he could have been a truly first-rate molecular biologist.

The medieval mind remains hale and hardy in many today and it continues to be resented by scholars in and out of the sciences. Bertrand Russell's (1945, p. 463) evaluation of Saint Thomas Aquinas deals with the medieval mind, whatever the period of its existence:

> He does not, like the Platonic Socrates, set out to follow wherever the argument may lead. He is not engaged in an inquiry, the result of which it is impossible to know in advance. Before he began to philosophize, he already knows the truth; it is declared in the Catholic faith. If he can find apparently rational arguments for some parts of the faith, so much the better; if he cannot, he need only fall back on revelation. The finding of arguments for a conclusion given in advance is not philosophy, but special pleading.

References to Medieval thought

Artz (1965, ch. 7), Haskins (1927, ch. 11), Knowles (1962), Taylor (1951, chs. 35–37).

THE REBIRTH OF NATURALISTIC THOUGHT

Slowly scholasticism revealed its inadequacy as a method of understanding man or nature and the inquisitive turned elsewhere. By the 13th century essentially all of Greek philosophy and science, with their fresh and open-ended procedures, became available to western scholars. Once the awe of the Greek accomplishment was overcome and the bondage to accepted authority had been broken, scholars could imitate what the Greeks did, not parrot what they said. Science became possible once again.

There was renewed interest in embryology. Leonardo da Vinci (1452–1519) both observed human embryos and left us some beautiful drawings of them and there are many other fragments of embryological observations and speculations. It is more realistic, however, to renew the narrative with Fabricius (1533?–1619) who for most of his active life was a crusty professor of medicine at the University of Padua and the teacher of William Harvey.

HIERONYMUS FABRICIUS OF AQUAPENDENTE

There was still the basic problem of trying to formulate questions that could be answered. This proved elusive so the study of development concentrated on what was possible—describing normal development. This is not an unworthy goal. Science seeks to associate and conceptualize the phenomena of nature. That activity, quite obviously, depends on knowing what the phenomena are.

Fabricius's *De Formatione Ovi et Pulli*, mainly about the chick, and *De Formato Foetu*, mainly about mammalian development, date from about 1600. Chicken eggs were opened daily after the beginning of incubation and the embryos were studied and drawn as in Figure 1—the earliest illustrations that have survived. No magnification was used (compound microscopes were just in the process of being invented). No wonder he said that in the four-day chick the body looks like a very tiny flea. By the fifth day, however, Fabricius could make out the head, eyes, heart, arteries, veins, liver, and lungs.

In this description of the developing chick, Fabricius combined the work of Aristotle and Galen with his own. When we remember that these three span nearly two thousand years, the advances made in embryology appear most modest. There is a strong Scholastic streak that makes Fabricius most reluctant to disagree with his illustrious predecessors, especially Aristotle. Nevertheless, he helped to keep alive an interest in the subject, he corrected some of the errors of Aristotle and Galen and adds a few of his own—such as believing that the embryo arose from the chalazae instead of the blastoderm, which he regarded as a scar representing the place where the egg attached to the ovary.

Those who studied embryology of the chick in the 15th–17th centuries were not biologists in the modern sense but physicians who were studying more convenient organisms in order to better understand human development. It is remarkable that they thought this was possible but, ages before, Aristotle and Galen had shown that the vertebrate embryos with which they were familiar all resemble one another to some degree. Therefore it seemed acceptable to study the chick, so easy to obtain, instead of the human embryo that was impossible to obtain in the early stages.

None of these observations led to any practical medical result so, one might ask, why was such work done? To put this question in perspective it must be remembered that very few individuals in the 16th and 17th centuries were so occupied. For those who were, however, attempts to gain understanding were most serious. They studied ancient authorities with care, made what observations they could, and speculated according to the canons of contemporary philosophy. They sought knowledge for its own sake. Progress was well nigh imperceptible—awaiting the tools and technology necessary to collect the necessary data and the addition to observation and speculation of the third element of basic scientific procedures—controlled experimentation.

The embryological treatises of Fabricius have been translated by Adelmann (*1942), who provides a biography and discussion of Ancient and Medieval embryology. The two volumes are a monument to scholarship and publishing. See also Meyer (1939) and Needham (1959).

But slowly the procedures of modern science were penetrating mind and laboratory. A colleague of Fabricius at Padua, Cesare Cremonini, wrote in 1596 that both teaching and learning must be based on "logic, with the opportune intervention of experience." The requirement for logic is obvious—there must be disciplined reasoning. Experience is necessary "because, though one be instructed by genius [*i.e.,* Aristotle] or by logic, unless he be also experienced in the very thing in which he is to judge, he will there exercise no judgment." But it is not always easy to make that opportune intervention of experience because "in the natural sciences such observation is not so obvious a way of gaining principles, nor is the collection of principles by its employment so easy. There is indeed required a laborious attention, procured from a zealous application to things;

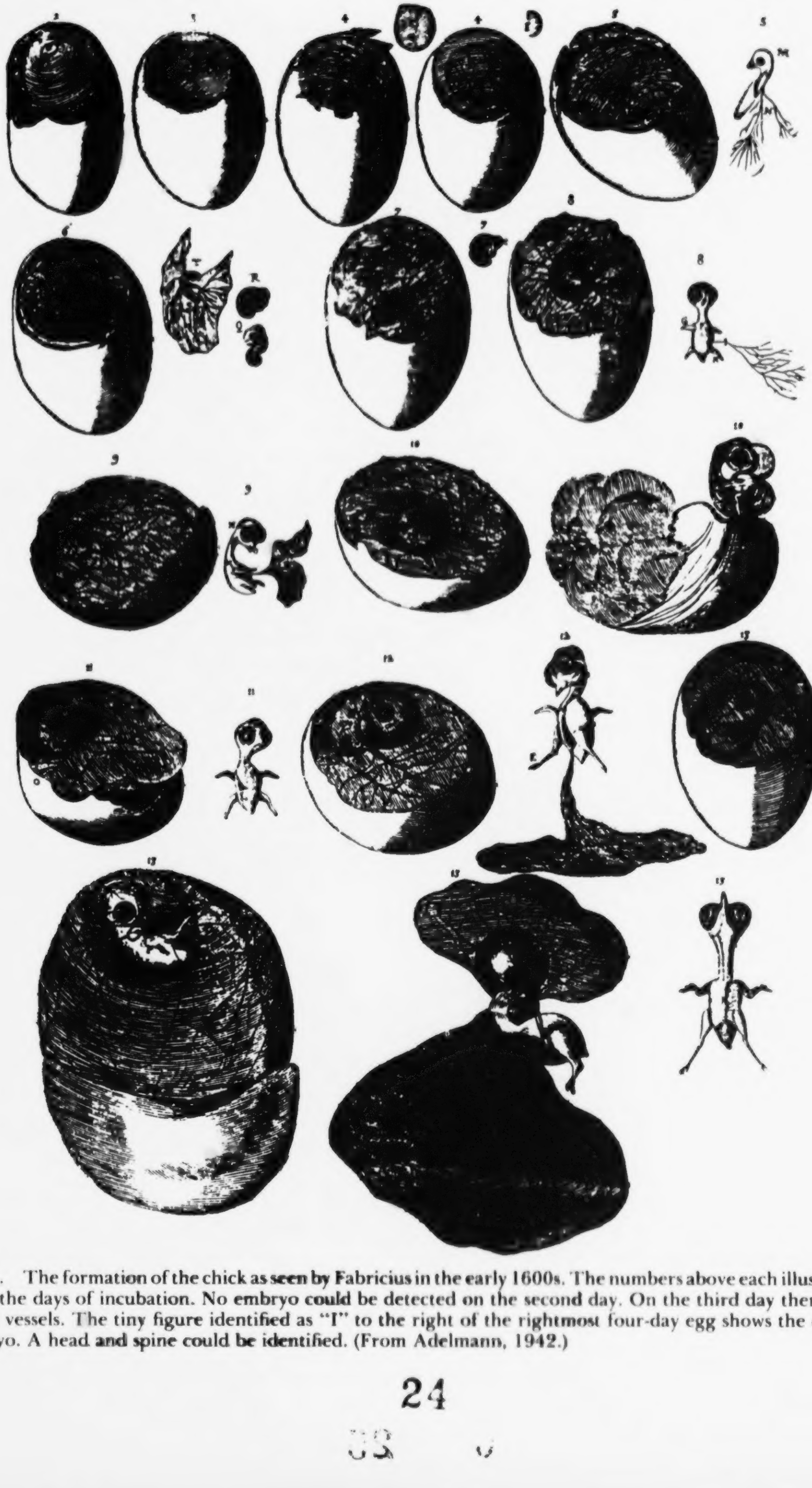

FIG. 1. The formation of the chick as seen by Fabricius in the early 1600s. The numbers above each illustration show the days of incubation. No embryo could be detected on the second day. On the third day there were blood vessels. The tiny figure identified as "I" to the right of the rightmost four-day egg shows the excised embryo. A head and spine could be identified. (From Adelmann, 1942.)

and even with it the principles are arrived at not without keen thought" (these quotations are from Randall, 1940, p. 204). Cremonini felt that progress was simpler in mathematics.

So the "zealous application to things" continued.

WILLIAM HARVEY

With the advent of William Harvey (1578–1657)—yes, the one concerned with the circulation of blood—there began

> the transition from the static to the dynamic conception of embryology, from the study of the embryo as a changing succession of shape, to the study of it as causally governed organization of an initial physical complexity (Needham, 1959, pp. 116–117).

Harvey was a student of Fabricius at Padua from 1598 to 1602 and this is where he first studied embryology. His *Exercitationes de Generatione Animalium* was published in 1651, half a century after his teacher's book on the same subject.

> Since our observations lead us to conclude that many things of great consequence are very different from what they have hitherto been held to be, I shall myself give an account of what goes on in the egg from day to day, and what parts are there transmuted, directing my attention to the first days especially, when all is most obscure and confused, and difficult of observation, and in reference to which writers have more particularly drawn the sword against one another in defence of their several discordant observations, which, in sooth, they accommodate rather to their preconceived opinions respecting the material and efficient cause of animal generation rather than to simple truth (p. 226; all quotes are from the 1965 edition).

Harvey then describes the development of the chick for each day up to the 14th, and in general, thereafter. Fabricius had believed the embryo to arise from the chalazae; Harvey correctly recognizes the origin as the blastoderm (to him the "cicatricula").

> As soon as the egg, under the gentle warmth of the incubating hen, or of warmth derived from another source, begins to pullulate [*i.e.*, to start to become a pullet], this spot forthwith dilates, and expands like the pupil of the eye, and from thence, as the grand centre of the eye, the latent plastic force breaks forth and germinates. This first commencement of the chick, however, so far as I am aware, has not been observed by any one (p. 229).

So, at last, we know where development begins. Harvey used a magnifying glass and that may have been the reason why he was able to make this discovery.

There follows a detailed account of the chick's development in which the observations and opinions of earlier students, mainly Aristotle and Fabricius, are confirmed, extended, and corrected. Thereafter he discusses more general matters where the cogency of the argument replaces direct observation.

For example, he rejects the old Aristotelian view that the female contributes only the substance (menstrual blood) and the male the effective generating stuff (male semen).

> For the egg is to be viewed as a conception proceeding from the male and the female, equally endued with the virtue of either, and constituting a unity from which a single animal is engendered (pp. 270–271).

After watching the daily changes in the developing chick, Harvey accepts epigenesis as the mode.

> The structure of these animals commences from some one part as its nucleus and origin, by the instrumentality of which the rest of the limbs are joined on, and this we say takes place by the method of epigenesis, namely, by degrees, part after part; and this is, in preference to the other mode, generation properly so called (p. 334).

That other mode seems restricted to insects where there is a conversion of a caterpillar into a butterfly

> already of a proper size, which never attains to any larger growth after it is first born; this is called metamorphosis. But the more perfect animals with red blood are made by epigenesis, or the superaddition of parts (pp. 334–335).

This hypothesis of epigenesis is strengthened by another belief that all parts of the body are derived from the same basic materials:

> For out of the same material from which the first part of the chick or its smallest particle springs, from the very same is the whole chick born; whence the first little drop of blood, thence also proceeds its whole mass by means of generation in the egg; nor is there any difference between the elements which constitute and form the limbs or organs of the body, and those out of which all their similar parts, to wit, the skin, the flesh, veins, membranes, nerves, cartilages, and bones derive their origin. For the part which was at first soft and fleshy, afterwards, in the course of its growth, and without any change in the matter of nutrition, becomes a nerve, a ligament, a tendon; what was a simple membrane becomes an investing tunic; what had been cartilage is afterwards found to be a spinous process of bone [a remarkable conjecture but based on what Harvey could see], all variously diversified out of the same similar material (p. 339).

A final important hypothesis of Harvey is that all life comes from eggs. The frontispiece (Fig. 2) of the 1651 edition of *De Generatione Animalium* shows Zeus opening an egg from which emerges a bird, human being, katydid, porpoise (?), deer, snake, spider, lizard, and various plants. An inscription appears on the egg: *ex ovo omnia.* This is usually expanded to *omne vivum ex ovo,* but that precise phraseology does not appear. "Exercise the Sixty-Second" carries the title "An egg is the common origin of all animals." It is clear, however, that Harvey is not using "egg" in the customary restricted sense since, quoting Aristotle, he accepts spontaneous generation as a mode of origin for some creatures.

> [These organisms] whether they arise spontaneously, or from others, or in others, or from the parts or excrements of these, have this in common, that they are engendered from some principle adequate to this effect, and from an efficient cause inherent in the same principle. In this way, therefore, the primordium from which and by which they arise is inherent in every animal. Let us entitle this the primordium vegetale or vegetable incipience, understanding by this a certain corporeal something having life in potentia; or a certain something existing *per se,* which is capable of changing into a vegetative form under the agency of an internal principle. Such primordia are the eggs of animals and the seeds of plants; such also are the conceptions of viviparous animals, and the worm, as Aristotle calls it, whence insects proceed: the primordia of different living things consequently differ from one another; and according to their diversities are the modes of generation of animals, which nevertheless all agree in this one respect, that they proceed from the vegetal primordium as from matter endowed with the virtue of an efficient cause, though they differ in respect of the primordium which either bursts forth, as it were, spontaneously and by chance, or shows itself as fruit or seed from something else preceding it. Whence some animals are spoken of as spontaneously produced, others as engendered by parents (p. 457).

Obviously the "efficient cause" is a critical element in Harvey's explanation. Here he is using the Aristotelian terminology, which recognized four causes: final, efficient, formal, and material. The *final cause* is the purpose of the object. The final cause of the chick embryo is to produce a chicken. Somehow the end was thought to influence the process—today we call this teleology and shudder at the notion. The *efficient cause* represents the underlying control. The efficient cause might be developmental mechanisms that control the chick's devel-

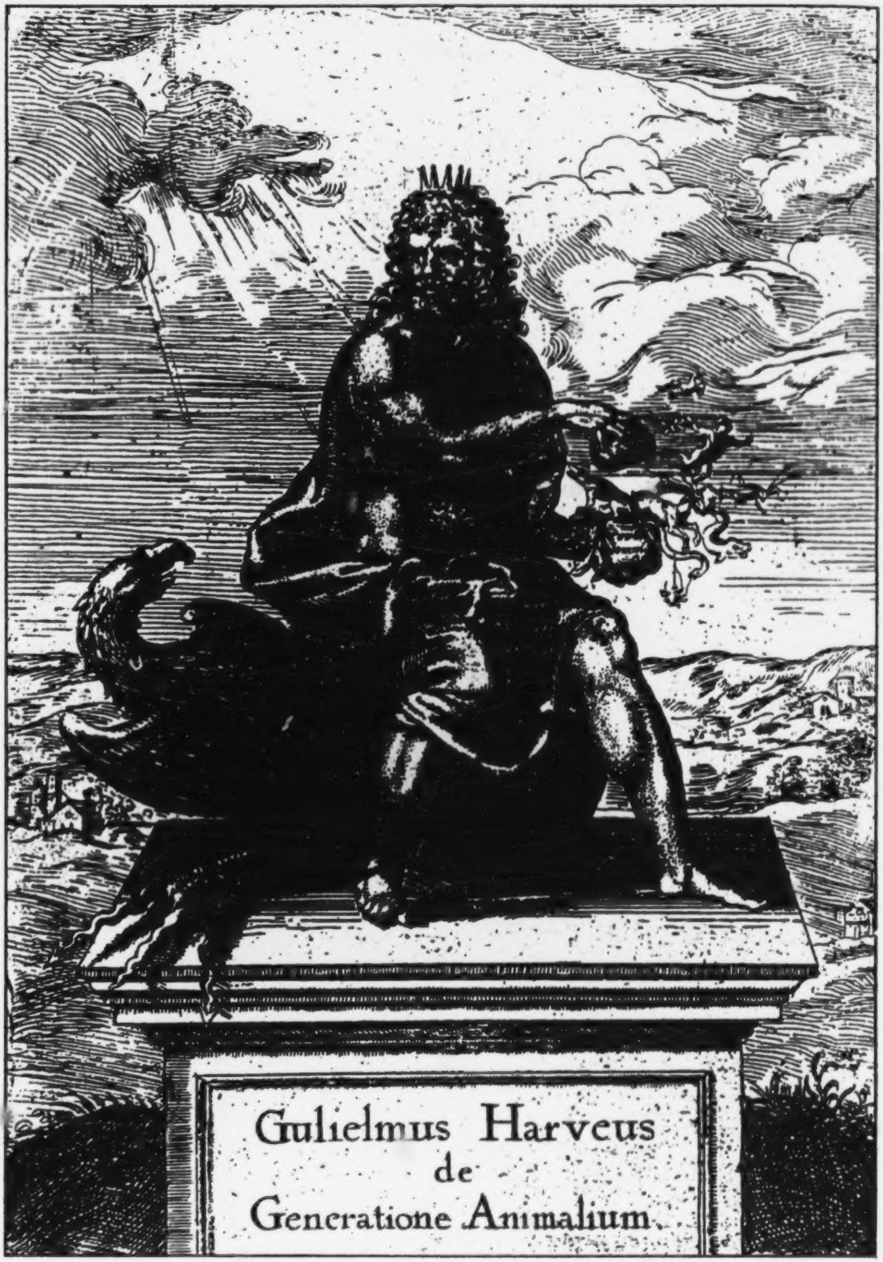
Gulielmus Harveus
de
Generatione Animalium

FIG. 2. The elegant frontispiece from William Harvey's (1651) *Generatione*.

opment. The *formal cause*, or form, may possibly be thought of as the DNA code that results in the embryo developing as, and becoming, a chicken. The *material cause* is the matter in the egg that is converted to the chick.

Aristotle's four causes have led to much confusion. The main problem is that scholars have failed to employ current words for Aristotle's ideas. Today only the "efficient cause" has even a remotely useful and modern meaning. (See Aristotle's *GA*, 715^a, 1–19 and especially the Introduction in Peck's translation, pp. xxxviii–xli.)

Thus when Harvey speaks of the "efficient cause" he means whatever it is that is controlling development. The nature of this efficient cause was an insoluble question for Harvey largely because he could not establish any continuity between material derived from the male and female and the offspring:

> Neither is there anything contained in the uterus immediately after intercourse, which, proceeding from the male, or from the female, or from both, can be regarded as the matter or rudiment of the future foetus (p. 356).

Thus he was demolishing Aristotle's hypothesis of menstrual blood and male semen as the contribution of parents to offspring. But something had to be the basis of genetic continuity, even where there seemed to be none, and Harvey proposed this hypothesis:

> So much is certain, and disputed by no one, that animals, all those at least that proceed from the intercourse of male and female, are the offspring of this intercourse, and that they are procreated as it seems by a kind of contagion, much in the same way as medical men observe contagious diseases, such as leprosy, lues venera [syphilis], plague, phthisis [tuberculosis], to creep through the ranks of mortal men, and by mere extrinsic contact to excite diseases similar to themselves in other bodies; nay, contact is not necessary; a mere halitus [breath] or miasma suffices, and that at a distance and by an inanimate medium, and with nothing sensibly altered: that is to say, where the contagion first touches, there it generates an "univocal" like itself, neither touching nor existing in fact, neither being present nor conjunct, but solely because it formerly touched. Such virtue and efficacy is found in contagions. And the same thing perchance occurs in the generation of animals (p. 358).

This was not a satisfactory solution and Harvey recognized as much. Embryology awaited Leeuwenhoek, von Baer, and Oscar Hertwig to establish the contributions of the parents. Furthermore, the fundamental questions that had concerned all embryologists from Aristotle to Harvey were to remain unanswered until controlled experimentation became possible and practiced. The field had to await George Newport, two centuries in the future. Harvey was a skillful experimenter in other fields, but not in embryology. According to Adelmann (1942, p. 121) Harvey

> in his work on generation did not escape the influences which mar the work of Fabricius; both were, in fact, deeply imbued with the spirit of the times in which they lived. Harvey built upon the foundations laid by Fabricius, and so in some cases approximated more closely the truth as we see the truth today; but Fabricius no less than Harvey contributed to its slow advance. Both struggled with problems far too difficult for their age to solve, but both contributed documents precious in the history of biological thought.

THE SCIENTIFIC REVOLUTION

So, once again, an embryologist provided better observations, corrected more errors, and sharpened speculation, but achieved no paradigm shift. Harvey lived during the early decades of the Scientific Revolution of the 17th century—a period of great intellectual ferment. The umbilicus to Aristotle was being severed, the world of nature was being accepted as fit for inquiry, science was becoming respect-able, observation and experimentation were replacing sole reliance on authority and deductive speculation, order was being discovered in the physical universe, censorship of Church and state was being challenged, theology was dethroned as the Queen of the Sciences, freedom of person and thought was increasing, theory and practice could be united in the same individual, universities were spreading, the Royal Society for the Improving Natural Knowledge was established (1662), the followers of Gutenberg (*ca.* 1400–1468) were hard at work, and the spread of prosperity increased the number of scholars who could work outside the Church.

There was no sudden springing to the barricades at the onset of the Scientific Revolution. In fact, there was no obvious beginning—only a slow spread of a new way of defining the methods of obtaining understanding of natural phenomena. Some historians date the onset of the Scientific Revolution at about 1660, near the time of the founding of the Royal Society of London (Burns *et al.*, 1986, pp. 861, 863). Such a date, however, seems odd since it excludes Vesalius, Harvey, Bacon, Copernicus, Galileo, Kepler, and Brahe and thus omits most of the intellectual giants who truly gave us science as a way of knowing. They are defined as the anticipators of the Scientific Revolution, and their exclusion leaves the period of the Scientific Revolution rather depopulated—since B. Russell (1945, pp. 525–526) writes, "Four great men—Copernicus, Kepler, Galileo, and Newton—are pre-eminent in the creation of science."

For reasons about to be mentioned my preference is 1543. No matter which birth date is selected we can speak of a "revolution" because some exceptional contributions to natural knowledge were made in a relatively brief time.

The year 1543 saw three key events in the history of science. One was the publication of *De Humani Corporis Fabrica* by Andreas Vesalius (1514–1564, a Belgian who became Professor of Anatomy at the University of Padua, where Fabricius later taught). Prior to this event Galen's anatomy was the authority. Vesalius was able to dissect human bodies and found that Galen was inaccurate in some instances. *De Humani Corporis Fabrica* has not only a complete description of gross anatomy but also beautiful illustrations by the Belgian artist Jan van Calcar (and not by Albrecht Dürer as has been suggested). This was the beginning of modern anatomy and is a straight path to Grey. Initially Vesalius had much opposition since even suggesting that such an ancient and respected authority as Galen might have erred was not in the best of taste. This opposition was primarily the attitude of the Church, which had a vested interest in the sanctity of (its) traditions and took severe measures to see that they were preserved.

A second accomplishment in 1543 was the publication of *De Revolutionibus Orbium Coelestrium* by the Polish physician, clergyman, and astronomer Nicholas Copernicus (1473–1543). Here is the beginning of modern astronomy. His hypothesis of heliocentrism lacked a convincing evidential base. It was only later that heliocentrism was made true beyond all reasonable doubt with the carefully collected data of the Danish astronomer, Tycho Brahe (1546–1601); and improvements in theory by the German astronomer, Johann Kepler (1571–1630); and the observations of the Italian physicist and astronomer Galileo Galilei (1564–1642). (Poland, Denmark, Germany, and Italy! science was truly international.)

The reaction of the church to this demotion of the earth, the site of God's creations, is well known. The Dominican monk, Giordano Bruno, who opposed all dogmatism and, in the main accepted the Copernican theory, paid for his intellectual independence by being burned at the stake in 1600. Luther had this to say about Copernicus "This fool wishes to reverse the entire science of astronomy; but sacred Scripture tells us that Joshua commanded the sun to stand still, and not the earth." Matters became truly serious after Brahe, Kepler, and Galileo had done their work. There was no escaping the conclusion that the earth rotates on its axis each day and circles the sun each year. Galileo had two trials by the Inquisition, and at the second in 1633 made a public recantation of his belief in the heliocentric theory.

Galileo's crime was less what he said about the movement of celestial bodies and more that he challenged the authority of the Church, which held that the earth was the center of the universe. There are three good reasons why Copernicus, who after all was the author of the theory that got Galileo into trouble, largely escaped the wrath of the Church: he was careful to say that his notion "was just a theory," he dedicated his *De Revolutionibus* to the Pope, and most importantly he died very shortly after its publication.

For details of Galileo's accomplishments and persecution, both so important in the progress of science, see Bernardini and Fermi (1965), Campanella (1616), Drake (1957, 1970), Galilei (1615), Kaplon (1965), McMullin (1967), de Santillana (1955), and Shea (1972). For Redondis' recent and controversial reinterpretation of the Galileo affair see Dickson (1986).

The third notable event in 1543 was the recovery, translation, and publication of the works of the Greek physicist and mathematician, Archimedes (287–212 B.C.). He had made astonishing contributions to mathematics and mechanics, and he was a notable inventor. He viewed the universe itself as a gigantic machine, operating on mechanical principles. This was a liberating notion in the 16th century when the forces of nature were thought mystical and probably unknowable. The mechanics of Archimedes was to be basic to the work of Galileo and then to that of Newton.

So it seems appropriate to start the Scientific Revolution in 1543 with Vesalius, Copernicus, and Archimedes. Their works and the spirit they engendered would be part of the intellectual climate of Fabricius and Harvey, whose work in embryology has already been discussed. (History remembers Harvey less for his work on the embryology of the chick and more for *Exercitatio Anatomica de Motu Cordis et Sanguinis* of 1628. According to Frank (1980, p. xii) this was "the single most important discovery in the history of the physiological sciences—the circulation of the blood." It was also the beginning of physiology as an exact science.)

Another notable event that was truly part of the Scientific Revolution but precedes its traditional date of onset—1660—is the works of Sir Francis Bacon (1561–1626): *The Advancement of Learning* (1605) and *Novum Organum* (1620). (A discussion of Bacon's work will be found in III, pp. 591–596.)

When we do reach the 1660s, finally, there are two initial events that were to have profound influences for embryology—the foundation of the Royal Society in 1662 (III, p. 608) and discovery of cells by the Englishman Robert Hooke in 1663 (III, pp. 608–610). The first was to stimulate the development of science itself and the second was to provide, nearly two centuries later, a more fundamental level of embryological analysis.

For many the climax of the Scientific Revolution is to be found in the works of the Englishman Sir Isaac Newton (1642–1727): *Philosophiae Naturalis Principia Mathematica* and *Opticks* (1704). His genius ranged from mathematics, astronomy, mechanics, and light to the laws of motion and gravitation.

From the death of Newton, there has been steady progress in science, and in quantity at least it seems to adhere to Galileo's law of acceleration. Science as a way of knowing was here to stay.

> The men who founded modern science had two merits which are not necessarily found together: immense patience in observation, and great boldness in framing hypotheses. The second of these merits had belonged to the earliest Greek philosophers; the first existed, to a considerable degree, in the later astronomers of antiquity. But no one among the ancients, except perhaps Aristarchus, possessed both merits, and no one in the Middle Ages possessed either (B. Russell, 1945, pp. 527–528).

We can turn to Henry Power (1623–1668) for an understanding of the high hopes of the Scientific Revolution. He had recently been made a member of the Royal Society and in the conclusion of his *Experimental Philosophy* (1664) he addresses those "generous Virtuosi, and Lovers of Experimental Philosophy" as follows:

> Certainly this World was made not onely to be Inhabited, but Studied and Contemplated by Man; and, How few are there in the World that perform this homage due to their Creator? . . . It is Reason that transpeciates our Natures, and makes us little lower than the Angels . . . . There is a world of People indeed, and but a few men in it; mankind is but preserv'd in a few Individuals; the greatest part of Humanity is lost in Earth, and their Souls so fixed in that grosser moity of themselves (their Bodies) that nothing can volatize them, and set their Reasons at Liberty . . . .
>
> And this is the Age wherein all mens Souls are in a kind of fermentation, and the spirit of Wisdom and Learning begins to mount and free it self from those drossie and terrene Impediments wherewith it hath been so long clogg'd, and from the insipid phlegm and *Caput Mortuum* of useless Notions, in which it has endured so violent and long a fixation.
>
> This is the Age wherein (me-thinks) Philosophy comes in with a Spring-tide . . . . Me-thinks, I see how all the old Rubbish must be thrown away, and the rotten Buildings be overthrown, and carried away with so powerful an Inundation. These are the days that must lay a new Foundation of a more magnificent Philosophy, never to be overthrown: that will Empirically and Sensibly canvass the *Phenomena* of Nature, deducing the Causes of things from such Originals in Nature, as we observe are producible by Art, and the infallible demonstration of Mechanicks: and certainly, this is the way, and no other, to build a true and permanent Philosophy (pp. 183, 184, 192).

And they did.

THE CONTRIBUTION OF VIVIPAROUS FEMALES

Apart from the possible research of Cleopatra, embryological studies from earliest times to the 17th century were the work of males. As such they were well aware of the male's contribution to conception but that of the female was confusing. Aristotle had recognized several patterns of generation (see Peck's translation of *GA*, pp. lxxii and lxxiii). Oviparous females like the hen laid eggs from which the young hatch. Ovoviviparous females such as sharks and some snakes have eggs but these were retained in the body until hatching. Females of human beings and other mammals, however, puzzled Aristotle and many who followed him. They thought that menstrual blood or some other secretion of the female contributed to conception. Harvey refuted this notion because he could find nothing in the uteri of deer after mating that might be the beginning of the new individual. Nevertheless it was assumed that the female must contribute something.

The answer seemed to come with some observations of de Graaf (1672) on the mammalian ovary, which at the time was called the *testis muliebris* (=female testis). Its function, if any, was unknown. Harvey thought that it had no role in copulation or generation. De Graaf found that some ovaries had spherical structures and he suspected they might be the long sought mammalian eggs or be "egg nests" (Sarton, 1931, p. 232). They came to be called Graafian follicles. This made it seem more reasonable, to many at least, that Harvey's dictum, *ex ovo omnia,* might be correct. Subsequently it was established by von Baer that Graafian follicles are not eggs but structures in which the eggs are formed.

MALPIGHI

Marcello Malpighi (1628–1694), an Italian biologist, followed Harvey by a generation. He was a professor at the University of Bologna for many years. His scientific contacts, however, were mainly with the Royal Society of London with which he corresponded actively—describing his latest discoveries in great detail and receiving encouraging letters from the Secretary.

The Royal Society published his two main works on the development of the chick (1672, 1675). They consist of minute descriptions of what he could see not only with the unaided eye but also with magnification—he was one of the first biologists to use the rapidly improving microscopes of the day. He had a variety of instruments and according to Adelmann (1965, p. 830) was able to obtain magnifications as high as 143×. Malpighi had no deep interest in causal factors and, in this sense, he contributed little. His descriptive embryology, however, was masterful.

Malpighi found that he could remove the blastoderm from a chick egg and place it on a glass slide. This simple technique, followed to this day, made it far easier to use the microscope in making observations. Such preparations remained useful even after drying. Apparently he did not take advantage of Robert Boyle's (1666) discovery that embryos could be preserved in Spirit of Wine (85+ percent alcohol) thus being available for study at more convenient times, or for comparing different stages of development, or even for demonstrating the drama of development to friends.

One important problem for Malpighi was to ascertain the structure of the chick embryo at the very beginning of development. This is what he observed (the illustration referred to is shown here in Fig. 3; Adelmann's 1965 translation is used, with his identifications of structures shown in brackets):

> In eggs laid the previous day and not yet incubated the cicatricula [blastoderm] (as I observed last August when the weather was very warm) was of the size I have roughly sketched in figure I,A. In the center of it there was found a cinereous [ash colored] saccule (B) [nucleus of Pander] that was sometimes oval, sometimes another shape. This saccule or follicle floated in the liquor of the colliquament (C) [blastoderm and area pellucida], which closely resembled molten glass and was confined in an irregular pit [subgerminal cavity], so to speak; for this colliquament was surrounded by a white ring of solid material (D) [germ wall] like an embankment, whose outer portion was bathed by a molten, limpid humor (E) [in area opaca]. Then followed a substance of little width (F) [area vitellina internal, often variously lancinated and likewise immersed in the humor (G). In addition, there were other, larger, surrounding circles (H), formed of the same more solid material and separated by channels of fluid (I). These outer circles (H), in particular, Nature does not form in one manner, and the material by which they are extended is not always continuous. Within the saccule, when I later held it against the sunlight, I noticed the fetus (L) enclosed as if in an amnion; and its head, with the first filaments of the *carina* [primordia of central nervous system] appended to it, was clearly evident. Indeed, the loose and diaphanous texture of the amnion [area pellucida] frequently permitted one to look through it and see the enclosed animal. I have often opened the follicle with the point of a needle to release the animal confined there, but to no purpose, for it was so mucous and so very tiny that in every case it was lacerated by a light touch. It is therefore proper to acknowledge that the first filaments of the chick pre-exist in the egg (pp. 941, 943, 945).

History has seized upon that last sentence to place Malpighi among the preformationists. If one assumes that an unincubated egg has not begun developing, the conclusion is inescapable that at least the beginnings of the chick's body are preformed in the "undeveloped egg." If at least some structures could be seen with the crude microscopes of the 17th century, it was reasonable to assume that much more was there awaiting discovery.

Adelmann (1965) has suggested that it is not necessary to conclude that Malpighi was a preformationist. Even in Malpighi's time it was suspected that whatever interactions occurred between the male and female contributions to the new individual took place while the egg was still in the body of the female. Thus, development *might* begin before incubation. Malpighi mentioned that the eggs he studied had been laid the previous day and the observations were made in "August when the weather was very warm." It would seem rather surprising for Malpighi to write about the weather when describing embryonic development unless he believed such metereological data were important. Undoubtedly they were. It is almost certain that considerable development had oc-

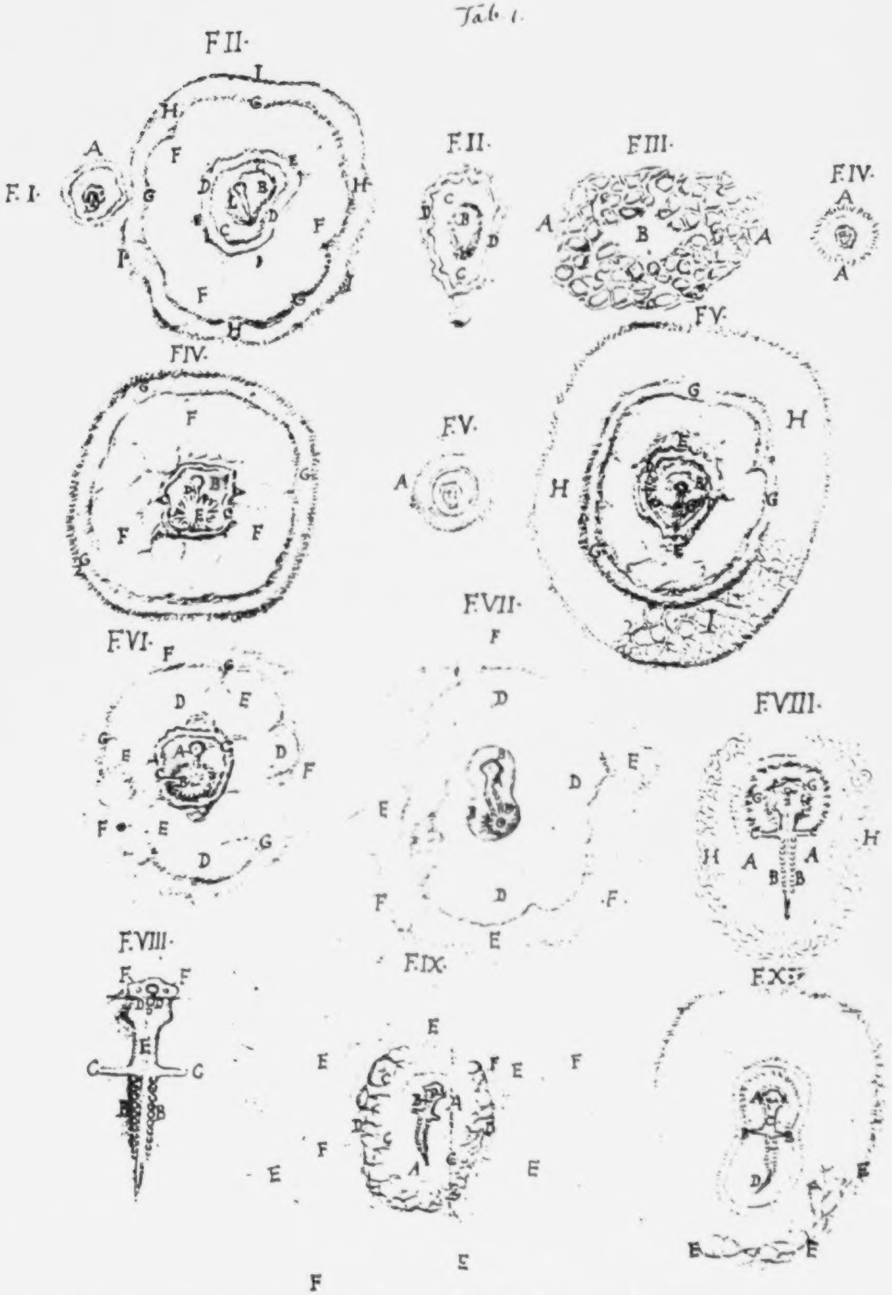
Tab. 1.
F.I.
F.II.
F.II.
F.III.
F.IV.
F.IV.
F.V.
F.V.
F.VI.
F.VII.
F.VIII.
F.VIII.
F.IX.
F.X.

FIG. 3. Malpighi's drawings of the early development of the chick. "F I" is an unincubated egg in which Malpighi thought he saw the beginnings of the embryo. "F II" is after 6 hours of incubation; "F IV" and "F V" after 12 hours. The oldest shown, "F X," is about what we would call a 40-hour embryo. (From Adelmann, 1966.)

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curred during that warm August day between the time the eggs were laid and Malpighi first studied them. A comparison of Malpighi's illustration of the unincubated egg with Huettner's (1941) figure 85 of an egg incubated 19 hours and figure 86, incubated 22 hours, suggests that a very warm August day can replace a brooding hen to some extent. This possibility is made more probable by the fact that the later stages described by Malpighi all seemed too advanced in terms of what later investigators found. (See also Hamburger and Hamilton, 1951, for an atlas of timed stages of chick development.)

Adelmann (1966) has provided us with an even more sumptuous and scholarly monograph on Malpighi than he did for Fabricius. It includes the originals and translations of Malpighi, a modern interpretation of what was known to Malpighi and other early students, a biography, and a remarkable account of the early history of embryology—a real *tour de force.*

* * * * *

Malpighi died in 1694 and the Royal Society, which had published his main works and corresponded with him for years, took note of the occasion by publishing an autopsy report (Phil. Trans. 19, pp. 467–471). This is quoted here as an aside—to give some notion of the state of biological science at the close of the 17th century.

> The Abdomen being opened, we found the Ventricle [here meaning the stomach], with the Guts, the Sweet-bread [pancreas], the Spleen and Liver, most sound, both as to colour and bigness; only the Bladder of the Gall abounded with a black Gall. The left Kidney had nothing amiss; but the right was twice as little, and had its *Pelvis* thrice as bigg; which discover'd the cause of the easie descent of the Stones. We found in the Bladder a little Stone, that seem'd to have fallen into it a few days before.
>
> When the Sternum was taken off, the Lungs appeared wither'd, with some mark of corruption on the backside. The Heart was bigger than ordinary, and the sides of the left Ventricle felt harder and thicker in some places than others; yet there was no Polypus found in either of the Ventricles, though there was ground to suspect it.
>
> At last the Skull being cut asunder, the true cause of his death was discovered, for the right Ventricle of the Brain contain'd almost two Ounces of extravasated Blood, and the left Ventricle was swell'd with a thick and yellow sort of Phlegm, which weigh'd more than an Ounce. Moreover the *Dura Mater* stuck closer to the Skull than is usual.

The advances in medical science had been sufficient by the end of the 17th century so that a proper diagnosis of the cause of Malpighi's death could be given.

> This proves that the conglobated Glands in the whole Body, had thrown into the Mass of Blood an Acid lymph, and that the conglomerated Glands of the Hypochondria [abdomen], especially those of the Liver had thrown into it a melancholy Humor, and that these two sorts of Humors being carried into the Vessels of the Brain, had dispos'd the Blood to coagulate there, and that having there corroded and broken the Tunicles [membranes] which serv'd for a stop to them, they had run into the Cavities, where they caused death without a Remedy.

Requiescat in pace.

A TWO MILLENNIAL SUMMING UP

The study of development can be divided into two main categories: descriptive and analytical. Until recently the first has been primarily a morphological discipline. The course of development, from conception to maturity, was described in detail—how the embryo (apparently) changes and grows. Included was the characterization of whatever it is that parents contribute to their offspring at the time of conception. Analytical or experimental embryology is concerned with the mechanisms of embryological change, *i.e.*, how whatever it is that parents contribute to their offspring is converted into a new individual. Thus descriptive embryology is "What happens?" and analytical embryology is "How does it happen?"

What had Aristotle, Galen, Fabricius, Harvey, and Malpighi accomplished in descriptive and analytical embryology? Not much—nor could they. Aristotle was the one most interested in concepts and causes—analytical embryology; Malpighi the least. At the conceptual level one could have passed directly from Aristotle to the 18th century and lost almost nothing. But the embryologists were not uniquely unsuccessful. Progress was slow in all fields of biology and, for that matter, in all fields of science. Notable progress was made in only some aspects of physics and astronomy.

There are valid reasons for this conceptual stasis. Concepts must be based on data and during those long millennia the necessary data were unavailable. The data were to come from a then invisible level of analysis—the level of cells and their parts. First there had to be microscopes and then came knowledge of cells. Microscopes, though inadequate, became available in the late 17th century. On April 15, 1663 Robert Hooke had reported to the Royal Society his observations on cells in cork (III, pp. 608–610) yet nearly two centuries were to pass before cells became important in embryological explanations.

There seemed to be a few general principles of development that were true beyond reasonable doubt. All were known to Aristotle and this is a measure of the lack of significant conceptual progress. Here is the balance sheet.

1. Sexual reproduction, the interaction of males and females, is required in many species. It was assumed that there must be some material contribution but it was not known what it might be.

2. Both sexes influence the characteristics of the offspring but the mechanism of this influence was not understood. This means that not only was the basis of genetic continuity a complete mystery but so also were the mechanisms of transforming that basis into a new individual of the same type as the parents. There was no clear distinction between transmission of material and transformation of that material into a new individual.

3. The embryos of different species of the same major group, birds for example, resemble one another closely. There are even resemblances among various species of vertebrates—mammals, birds, and fishes.

4. Development appeared to be epigenetic, although Aristotle and later workers were unsure since microscopes were not adequate to show any minute beginnings.

Clearly the Scientific Revolution did not produce any vast improvement in the understanding of development. In fact, its effect on the life sciences as a whole was slight. Vesalius produced a better human anatomy than Galen's but no conceptual breakthrough was involved. Physiology started grandly with Harvey's observations and experiments on circulation, but thereafter progress was exceedingly slow.

One could argue that a knowledge of embryology did not have a high priority among scholars at that time and hence progress would be slow and episodic. True enough—there never was a critical mass of individuals concerned with development during those millennia.

But this cannot be the entire explanation since the same argument does not apply to medicine. There had always been many individuals with a deep concern for learning about human ailments and how to ameliorate them; yet progress was slow and seemingly the physicians were as perplexed as the embryologists at the end of the 17th century.

The following quotation, by an English physician Dr. James Cooke (1762), illustrates how much ideas in biology would have to change before modern understandings were to be possible. Cooke was a preformationist and an animalculist—one who believed that an already-formed body was located in the sperm, or animalcule. He was concerned with the fate of all those sperm which were present in semen but were not to be involved in conception:

> All those other attending Animacula, except that one that is conceived, evaporate away, and return back into the Atmosphere again, whence it is very likely they immediately proceeded; into the open Air, I say, the common Receptacle of all such disengaged minute sublunary bodies; and do there circulate about with other *Semina*, where, perhaps

> they do not absolutely die, but live a latent life, in an insensible or dormant state, like Swallows in Winter, lying quite still like a stopped watch when let down, till (they) are received afresh into some other male Body of the proper kind . . . to be afresh set on Motion, and ejected again in Coition as before, to run a fresh chance for a lucky Conception: for it is very hard to conceive that Nature is so idly luxurious of Seeds thus only to destroy them, and to make Myriads of them subservient to but a single one (quoted from Punnett, 1928, p. 506).

Not everyone would have accepted Cooke's analysis but this quotation suggests that the Middle Ages were alive and well in his thought patterns in the late 18th century. This quotation shows how much, of necessity, must be unlearned before progress was to become possible.

During the Scientific Revolution notable progress in conceptual science was being made only in the case of those natural phenomena that could be studied quantitatively. For example, primitive human beings had long observed the motions of stars and planets and had developed impressive predictive abilities. This line of analysis was extended in the Scientific Revolution when the data were used by Copernicus and Kepler. Data that suggested the laws of motion to Galileo and Newton were relatively easy to obtain, yet required genius to interpret, as were those that led Robert Boyle to see the relation of volume to pressure in gases. Newton's theory of gravitation, an undisputed stroke of true genius, again dealt with relatively simple relationships.

Progress in the separate sciences was to be, in a general way, inversely related to the complexity of the phenomena they were attempting to conceptualize and directly related to the ease with which relationships could be expressed in mathematical terms. Far more work had to be done before biology could enter a period of impressive and sustained progress.

It is important that students recognize that science can remain in a relatively sterile period such as those two millennia from Aristotle to Malpighi. Progress in science is usually presented to them as a series of consecutive discoveries that, if the time scale is omitted, suggest rapidity and inevitability. This is not so. Consider that most elegant feature of the Scientific Revolution, Newton's Theory of Gravitation. Once it had been formulated and applied to various phenomena, progress seemed to cease. Physicists today are still struggling to think further about gravity—what is "it" that seemingly pulls bodies towards one another in relation to their mass and distance apart. We know, most precisely, what gravity can do—not what it is.

Thus it should be noted that the seemingly inexorable advance of science is not a reflection of continuous progress in solving problems but of one advance now, another later. Progress should not be visualized as a host of parallel arrows but as a network with a very irregular advancing edge. One small area of that edge will be pushed out and only gradually will some of the adjacent areas be "pulled along." Progress in cytology and Mendelian genetics slowed until it was discovered that the data of one provided deep understanding of the other (III, pp. 653–660). Attempts to determine the age of the geological strata reached a stalemate until an advance in an entirely different field, radioactivity, provided new techniques and insights (I, pp. 487, 491–492, 513). Direct attempts to determine the nature of genes reached a dead end and further progress depended on advances in biochemistry. And embryology remained in an eddy until the equipment and techniques for studying cells became available.

One might say that embryologists were floundering. In fact, most scientists of all persuasions were. The early volumes of the *Transactions of the Royal Society* list the things considered at each meeting and these show the very elementary nature of the discussions and concerns. Those relating to biology were as follows: a description of an abnormal calf with no joints in the hind legs and with a three-parted tongue; Hooke's description of the appearance of many things under his microscope, including a slice of cork; a test on whether or not one was unduly thirsty after eating viper flesh; suggestions for protecting ships' bottoms from being eaten by nauphagous ("ship-eating") worms; raising silkworms in Virginia; how to kill ratle-snakes in Virginia; the presence of shining worms in oysters; transfusions of blood between dogs and the suggestion that it would be good to know "whether those dogs, that have peculiarities, will have them either abolisht, or at least much impaired by transfusion of blood."

Yes, scientists were floundering but surely this is a necessary stage in the passage from ignorance to understanding. Floundering, at the very least, indicates activity and concern.

PREFORMATION AND EPIGENESIS

The resolution of the conflicting hypotheses, preformation or epigenesis, was the dominant theoretical problem of embryologists from the last quarter of the 17th century to the end of the 18th. This was also the first time that a sufficient number of individuals, a critical mass, was alive at the same time and so could engage in dialogue. One could now argue with the living instead of solely with the dead—a process of enormous importance in resolving issues, detecting errors, comparing techniques, and making scholarship seem worthwhile. Science is a social enterprise, obviously so today, thus it is necessary to have enough practitioners at any one time to interact effectively.

In its most restricted sense, preformation means that the parts of the adult exist as such, albeit much smaller, at the very beginning of development. Some preformationists, also known as "evolutionists," reported that they could see tiny organisms in eggs or in sperm. Although there is some doubt about Malpighi's position, as noted before, the following quotation from *De Formatione Pulli in Ovo* seems to represent the preformationist position:

> [When] studying attentively the genesis of animals from the egg, lo! in the egg itself we behold the animal already almost formed, and our labor thus is rendered fruitless. For, being unable to detect the first origins, we are forced to await the manifestation of the parts as they successively come to view (Adelmann, 1966, pp. 935, 937).

In epigenesis, on the other hand, the adult parts are not present at the beginning of development but appear seriatim as development proceeds even though the earliest stages of development can not be seen. Some embryologists, from Aristotle to Harvey, believed that epigenesis was the more probable hypothesis. Since neither hypothesis could be proven to be true beyond all reasonable doubt, cogent argument became the main method for defending one's position.

Today we tend to regard this effort to prove beyond all reasonable doubt that one or the other hypothesis is correct as possibly charming but probably silly. Neither is true. Those who debated preformation *vs.* epigenesis were concerned with the fundamental problem of differentiation. How could structures appear in the course of development from structureless material? What could be the stimulus that would convert structureless semen into heart, brain, legs, eyes and all the complex parts of the body? A 5th century B.C. Greek philosopher-scientist, Anaxagoras of Clazomenae (in Ionia), and some other philosophers, held that truly new things cannot originate. There could be no "coming-into-being out of non-existence" as Cornford (1930, p. 30) expresses it. Cornford quotes an ancient commentator who was not impressed with this view of Anaxagoras:

> Anaxagoras, finding an old doctrine [that of Parmenides] that nothing comes into being out of what in no way is, abolished coming-into-being and substituted for it a process of becoming distinct. He talked nonsense about all things being mixed with one another and becoming distinct as they grow. For in the same germ, he said, there are hair, nails, veins, arteries, sinews, bones. These are present in particles so small as to be invisible, but as

> they grow they gradually become distinct. '*For how,*' he says, '*could hair come out of non-hair or flesh out of non-flesh?*'

The hypothesis of preformation circumvented the problem of differentiation—structure was present from the very beginning so there was no problem of deriving form from a formless beginning. Preformationism was based initially on an inability to see how epigenesis might work. Epigeneticists on the other hand based their hypothesis on observations, crude as they were, that seemed to show that new things did appear during development. Moreover, they were able to advance objections to preformation as in the case of hybrids. If the egg of a horse contained a preformed horse, how could one account for a mule? When different varieties of plants are crossed, how can the offspring be intermediate? If there is a strict preformation, how can there be any variation among offspring at all if they are raised under the same conditions?

But pure epigenesis also raised serious problems. One could argue that there must be some sort of preformation in the sense of there being a transfer of "information." Offspring do resemble their parents—rabbits do not hatch from hen's eggs. This transfer of information could be imagined to occur either at conception or later in the viviparous species. In oviparous species, however, especially those that broadcast their semen into the ocean, there could be no subsequent transfer of information from parent to offspring. So if there was some general rule that applied to all species, the transfer of information must occur at conception. Thus there must be preformed information whether or not there were preformed structures.

Therefore preformation *vs.* epigenesis is far from a trivial problem. It confounded philosophers from the earliest times and remained unresolved as the 17th century came to a close. Next we will trace how it was dealt with during the 18th century by those who came after Malpighi.

An aside. From now on we will use the terms "ova," or eggs for the female's contribution to the young and "sperm" for the male's contribution. In 1667 Leeuwenhoek reported that animalcules, later spermatozoa, were present in semen and suggested they were the active agent (III, pp. 614–615). Somewhat earlier, in 1651, Harvey had suggested that all life comes from eggs. Neither view was accepted by all until more than a century passed, but to avoid circumlocution, from here on these terms will be used.

DEDUCTIONS FROM THE HYPOTHESIS OF PREFORMATION

That profound philosophical difficulty of Parmenides, Anaxagoras, and later preformationists of how there could be a "coming-into-being out of non-existence" caused most embryologists of the late 17th and the entire 18th centuries to reject epigenesis.

However, some of the deductions from the hypothesis of preformation proved exceedingly troublesome. For example, if we assume that both ova and sperm have preformed bodies one might deduce that twins would result from each conception. But this is not true so how could one account for a single offspring? Could one imagine the fusion of two little heads, hearts, skeleton, and all the other complex parts of the body? One had to assume some sort of amalgamation otherwise twins should be the usual occurrence in human births.

This difficulty was circumvented with the assumption that *either* the sperm or ovum would contain the tiny body. In the case of human beings a homunculus, or "little man" was predicted. Not surprisingly this resulted in two schools of thought among the preformationists: the ovists who believed the homunculus to be only in eggs and the spermists (or animalculists) who believed the homunculus to reside in sperm. These were not silly aberrations of human thought but necessary deductions from the hypothesis.

How could these deductions be tested? By looking. There was a severely restricted problem here for the ovists. The true mammalian egg was not to be discovered until 1827, by von Baer. The spermists did not suffer this restriction. Leeuwenhoek had reported that semen contains microscopic animalcules, later to be given the name "spermatozoa" by von Baer. These were examined with the crude microscopes of the day and, as predicted, found to contain tiny bodies. It was a necessary deduction, of course, that the tiny bodies in sperm would be species specific. And they were. Gautier d' Agoty (1750) claimed to see tiny chickens, horses, and donkeys in the semen (not sperm) of those species. Earlier Hartsoeker (1694) had provided an illustration of a severely cramped homunculus with a huge head and the fontanelle clearly indicated (Fig. 4). Hartsoeker made no claim that he had observed this homunculus—merely that if he could see it that is what it would look like. Others described sperm as being of two sorts—some with a male homunculus and others with a female homunculus.

These observations, or better imaginations, were not accepted by all—not even by some of the strict preformationists. The absence of acceptable verification, however, was not necessarily a serious problem. The fact that simple microscopes and simple techniques had revealed a rich and previously unseen world suggested that surely improved technology would greatly expand that world.

During the 17th and 18th centuries information about regeneration began to become available. Some animals were found to have astonishing abilities to replace lost parts. Strict preformationism, however, would preclude the possibility of regeneration. Yet it occurred and that knowledge led Hartsoeker to abandon preformationism.

Another deduction from preformation was so necessary and so improbable to many that it contributed to the rejection of the hypothesis. Let us adopt the ovists position and assume that the human egg has a completely formed homunculus—of a female. Then that homunculus must contain ovaries and those ovaries must have eggs with homunculi. Those homunculi again must have the next generation of homunculi and so on—like a set of Russian dolls. This deduction is a logical necessity from the hypothesis of preformation since the possibility of anything new appearing (epigenesis) is excluded.

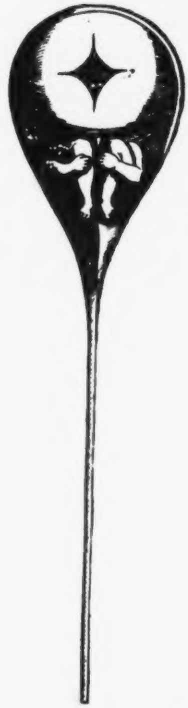


FIG. 4. Hartsoeker imagined that the human sperm might look like this. This is not his observation but his hypothesis (from *Essai de dioptrique*, Paris, 1694).

One cannot imagine an infinite series of ever smaller and ever encased homunculi, so eventually the supply would be exhausted and the species would become extinct. It was suggested that the entire future of the human race was included in the successively encased homunculi in the ovaries of Eve. In more senses than one, the ovists thought of her as the Mother of Humanity.

This strict preformationist position was adopted by Malebranche (1672) late in the 17th century and continued with von Haller (1767) and Bonnet (1770) in the 18th century when it was the dominant hypothesis, and was given the name *emboîtement* ("encasing").

THE EPIGENESIS OF EPIGENESIS

The hypothesis of preformation accounted for a very great deal but it could not account for everything. Slowly efforts to refute it gained ground. In 1759 Caspar Friedrich Wolff published his *Theoria Generationis* based mainly on the chick. Wolff interpreted his observations as indicating a true epigenetic development. He worked in Germany, so presumably his eggs did not have to endure those hot August days that Malpighi mentioned. He observed embryos at a much earlier stage than Malpighi and saw no recognizable organs. Preformationists such as von Haller countered once again that just because structure could not be seen one could not conclude that it was not there.

But Wolff did make one strong and eventually convincing point. He emphasized that when organs first become clearly observable they are not in their final form. For example, the intestine of the chick embryo could be shown to start as a flat sheet and then become a tube. Epigenesis, therefore, was proven for individual structures. Thus is was not unreasonable to extend the hypothesis to development as a whole.

Other observations, such as those on plant and animal hybrids, could be accounted for more satisfactorily by the hypothesis of epigenesis than by that of preformation. Hybrids are generally intermediate. The hybrid individual would have been derived from the egg (assume the ovist position) of one type and, so, should not show any features of the other type.

In addition, how could preformationism account for variability within species? If Mother Eve really did have all the entombed members of humanity for all time in her ova, how could one account for the existing human races?

It might be interesting to see how your students evaluate the two hypotheses with the information given so far.

Then there was the seemingly well-established fact of spontaneous generation already mentioned in the discussion of Harvey. From the Greeks onward it was generally held that some organisms arose spontaneously—in decaying meat, from excrement, and in decaying food. Although in the century before Francesco Redi (1668) had made what were later to be regarded as definitive experiments, spontaneous generation of at least some lowly creatures was still accepted as "fact." Now, if organisms as complex as insects can arise spontaneously, the hypothesis of preformation becomes difficult to maintain. One cannot imagine that all meat contains preformed primordia of insects, which will start to develop once the meat begins to decay.

By the end of the 18th century epigenesis was slowly replacing preformationism as the dominant hypothesis. Embryologists were able to make better observations and the preformationist argument that "just because you can't see it doesn't mean that it is not there" carried less weight. Epigenesis seemed to explain more and require fewer improbable deductions. That deduction about Eve's ovaries was hard to accept as probable by those touched by the Enlightenment of the 18th century. If one ceases to believe in Eve, as many did, the demographic aspects of her ovaries do not present an insuperable problem.

The whole debate was, according to Sarton (1931, p. 317), a waste of time because

> the fine observational tradition of the seventeenth century [was] interrupted, or at any rate considerably slowed down for more than a century by discussions which were irrelevant, because they were too far ahead of the experimental data.

So, if we accept epigenesis, the awesome problem of differentiation still remains. The structure of embryological theory at the end of the 18th century remained roughly as it was formulated by Aristotle.

References: Galen to Wolff

Embryology. Adelmann (1942, *1966), Baxter (1976), Belloni (1974), Bowler (1971, 1973), Bylebyl (1972), Castellani (1973), Churchill (1970*a*), Cole (1930), Cooke (1762), Farley (1977, 1982), Gaissinovitch (1978), Gasking (*1967), Glass (1959), Haeckel (1905*b*, ch. 2), Harvey [1982], Heniger (1973), Highmore (1651), Hintzsche (1972), Maienschein (1986), Meyer (1936, 1938, 1939), Moore (1972*a*,

1972*b*), Needham (*1959), Oppenheimer (1955, *1967, 1975), Pagel (1967), Pilet (1970), Preus (1977), Punnett (*1928), Roe (1975, 1979, 1981), E. S. Russell (1917, *1930), Sandler (1973), Sarton (1931), Wallace (1970), Webster (1967), Wheeler (1899), Whitman (1896*a*, 1896*b*, 1896*c*), L. G. Wilson (1972), and Zanobio (1971).

Biology and science in general. Birch (1756–1757), Bodenheimer (1958), Butterfield (1957), Crombie (1952, 1953, 1959, 1963), Dampier (1929), Gardner (1965), A. R. Hall (1954), Haskins (1924), Hooykaas (1972), Koyré (1957, 1973), Lanham (1968), Locy (1915, 1925), Magner (1979), Mason (1953), Mayr (*1982), Mazzeo (1967), Nordenskiold (1928), Palter (1961), Radl (1930), Ritterbush (1964), B. Russell (1945), Sarton (1927–1948, 1952), Singer (*1950, *1959), Sirks and Zirkle (1964), de Solla Price (1975), Sprat (1722), Taton (1963), Thomson (1899), Thorndike (1923–1941), Whitehead (1925), Wightman (1951), and Woodger (1929).

The 1800s—Century of Discovery

We now cross a most important time-line in the development of science and enter the 19th century. This will be the century when humanity first began to truly understand, control, and predict the phenomena of science and nature. In roughly the first half of the century chemistry was to have its John Dalton (1766–1844), geology its Charles Lyell (1797–1875), and biology its Charles Darwin (1809–1882)—all Englishmen. There were to be radical changes in these three sciences whereas astronomy and physics, already with notable accomplishments, were to continue their rapid evidential and conceptual development.

The early 19th century was also to witness radical and irrevocable changes in the ways that people live. James Watt's (1736–1819) perfected steam engine of the late 18th century powered the Industrial Revolution and was the basis of George Stephenson's (1781–1848) locomotive engine. Again, both Watt and Stephenson were Englishmen. The arts of technology and transportation were unleashed.

Life in Western Civilization could never be the same again after the early 19th century. In field after field the impossible became possible—leading to the ultimate "impossibilities" of our own times. The renowned historian Fernand Braudel (1985) describes the crossing of this time boundary:

> Can it not be said that there is a limit, a ceiling which restricts all human life, containing it within a frontier of varying outline, one which is hard to reach and harder still to cross? This is the border which in every age, even our own, separates the possible from the impossible, what can be done with a little effort from what cannot be done at all. In the past, the borderline was imposed by inadequate food supplies, a population that was too big or too small for its resources, low productivity of labour, and the as yet slow progress in controlling nature. Between the fifteenth and the eighteenth century, these constraints hardly changed at all. And men did not even explore the limits of what was possible.
>
> It is worth insisting on this slow progress, this inertia. Overland transport, for example, very early possessed the elements which could have led to its being perfected. And indeed here and there, one finds faster speeds being reached because modern roads were built, or because vehicles carrying goods and passengers were improved, or new staging-posts established. But progress of this kind only became widespread by about 1830, that is just before the railway revolution. It was only then that overland transport by road became commonplace, regular, well-developed and finally available to the majority; so it was only then that the limits of the possible were actually reached. And this is not the only area in which backwardness persisted. In the end, the only real change, innovation and revolution along the borderline between possible and impossible came with the nineteenth century and the changed face of the world (p. 27).

And so it was with developmental biology though it was not until later in the 19th century that rapid and sustained progress in the "how's" of development became possible. The first few decades saw a few outstanding embryologists building on the discoveries of previous workers and using the slowly improving technology of the time to make notable advances in descriptive embryology–the chick embryo continuing to be the material of choice. There were no startling breakthroughs and no radical new theories that directed research programs in new ways.

ANOTHER "FATHER" OF EMBRYOLOGY—VON BAER

One of these embryologists was Karl Ernst von Baer (1792–1876) an Estonian biologist. He and others of his time, such as his colleague, Heinrich Christian Pander (1794–1865) a Latvian, began to study chick embryos in better ways. Malpighi's method of removing the early embryo from the egg and placing it on a glass slide for study continued to be used, together with Boyle's suggestion for preserving (="fixing") embryos with alcohol or other substances. A method perfected by botanists for making thin slices of tissues with a very sharp razor was also employed. These thin slices, mounted on slides and studied with a microscope, revealed structures that could not be seen in whole mounts. This was long before microtomes were available and the slices were made freehand. That was not so difficult with plant stems, roots, and leaves for example, because of their rigid cell walls. It is not simple, however, to make very thin slices of a chick embryo even after fixing in alcohol. (Paraffin embedding was half a century in the future.)

Von Baer's main contributions are to be found in two monographs, *De Ovi Mammalium et Hominis Genesi* (1827) and *Über Entwicklungsgeschichte der Thiere: Beobachtung und Reflexion* (1828; a second, and unfinished, volume was published in 1837). When reading these next few pages remember that another decade would pass before the cell theory was applied to animal tissues.

Von Baer begins *De Ovi Mammalium et Hominis Genesi* with a discussion of earlier attempts to discover something in mammals that corresponded with the well known eggs of birds, fishes, reptiles, amphibians and many invertebrates. These eggs were large and readily visible. Where could the mammalian egg be, or did it really exist? In von Baer's time this was the state of theory and knowledge. Interest centered on the structure earlier described by de Graaf (see also Corner, 1958, ch. 10).

> It seems beyond question that the Graafian vesicle contributes something to the development of the ovum, because after conception it is changed into the corpus luteum. Among anatomists today two opinions prevail regarding the manner in which the ova arise from the vesicles. Some believe that the Graafian vesicles correspond exactly to the yolk of birds' eggs, and that in innate fluid surrounds the little membranes, and therefore that they are received from the tube in the form of an egg. Previously I myself supported this opinion, because of the conspicuous similarity between the ovaries of mammals and of birds as well as because of the development of the foetus. Others believe that the fluid of the vesicles is ejected, and that it forms the ovum in the tubes either by itself or mixed with male semen (O'Malley, 1956, p. 122).

Von Baer started with the known and then sought the unknown. That is, he began by studying early embryos in the uterus and then sought even earlier stages in the oviducts. Presumably the earliest eggs in the oviducts would resemble something in the ovary. If he discovered them, that would complete the link.

> When I examined the ovaries before incising them, I clearly distinguished in almost all the [Graafian] vesicles a whitish-yellow point which was in no way attached to the covering of the vesicle, but as pressure exerted with a probe on the vesicle indicated clearly, swam freely in its liquid. Led on more by inquisitiveness than by the hope of seeing the ovules in the ovary with the naked eye through all the coverings of the Graafian vesicles, I opened a vesicle, of which, as I said, I had raised the top with the edge of a

> scalpel—so clearly did I see it distinguished from the surrounding mucus—and placed it under the microscope. I was astonished when I saw an ovule, already recognized from the [Fallopian] tubes, so plainly that a blind man could scarcely deny it. It is truly remarkable and astonishing that a thing so persistently and constantly sought and in all compendia of physiology considered as inextricable, could be put before the eyes with such facility (O'Malley, p. 132).

Von Baer undertook to repeat these observations on other species—after all he was searching for the *mammalian* ovum. He was able to report in the 1827 monograph that he had

> compared the [Graafian] vesicles of cows, sheep, dogs, rabbits, the stag, porpoise and dolphin, as well as man, with [pigs], and I have persuaded myself that in all of these animals the structure is the same (O'Malley, pp. 134–135).

The 1827 monograph contains the first generally accepted description of the long sought mammalian egg but, as is frequently the case, discovery involves some error. This was his conclusion:

> *When we take the ovary and in general the maternal organism into consideration, the Graafian vesicle is thus the real egg of mammals.* However, as far as its development is concerned, it diverges widely from the egg of other animals; these are carried intact out of the ovary, and they not only provide for the fetus a place of development, they transform themselves into that fetus. In the mammals the *embedded vesicle* contains a more developed yolk and *behaves with regard to the coming embryo as the real egg. It might be called the fetal egg within the maternal egg.* The mammals have thus an egg within an egg, or, if this way of putting it may be allowed, an egg in the second power (Sarton, 1931, p. 322).

Sarton (p. 320) mentions von Baer's emotion at the moment of discovery:

> When he first saw the minuscule sphere of yolk which was the egg he had been dreaming of, he was so struck that he was obliged to rest himself before he had the courage to look a second time into his microscope; he was afraid of having been deluded by a phantom.

It was realized later that the true ovum is not the entire Graafian follicle but the much smaller structure within it. We can easily identify von Baer's "egg within the egg" as the mammalian egg and wonder why he did not reach the same conclusion—especially when he realized that the Graafian follicle remained in the ovary and became the corpus luteum. Sarton explains why (1931, p. 324):

> A man makes a great discovery and misinterprets it because he is hypnotized by earlier ideas. The history of science is full of similar examples. It shows that the most difficult thing of all is to see things as they are, without preconception. Few people are able to do it at all, and these few only by intermittence.

Von Baer's full page illustration is shown here as Figure 5. Figure IX shows the "Vesicula Graafiana" of a breeding sow enlarged ten times (reduced slightly from this in Fig. 5). The tiny spherical structure at the top, identified by line 8, is the "ovulum." After the ovum has been extruded the Graafian follicle becomes the corpus luteum. Figure XIV, immediately below shows the corpus luteum of a dog.

In the upper dark band is the large number "1" and just below it is a tiny white dot, which is a small ovarian egg. Below "2" is a mature egg. Both are from a dog and are *natural size.* Then come the truly exciting discoveries. Figure 3 is a dog's egg from the oviduct and Figure 4 is one from the uterus. Again these are natural size. The illustrations I–III immediately below the dark band are the same eggs magnified ten times and in I*–III* they are magnified 30 times.

Later stages are also shown. Figure 6 is a dog embryo of 12 days, again natural size. The beautiful drawing of Figure VII^a is also of a dog embryo. The heart, ventral aorta, four aortic arches, and dorsal aorta are clearly shown as are the brain, eye, ear,

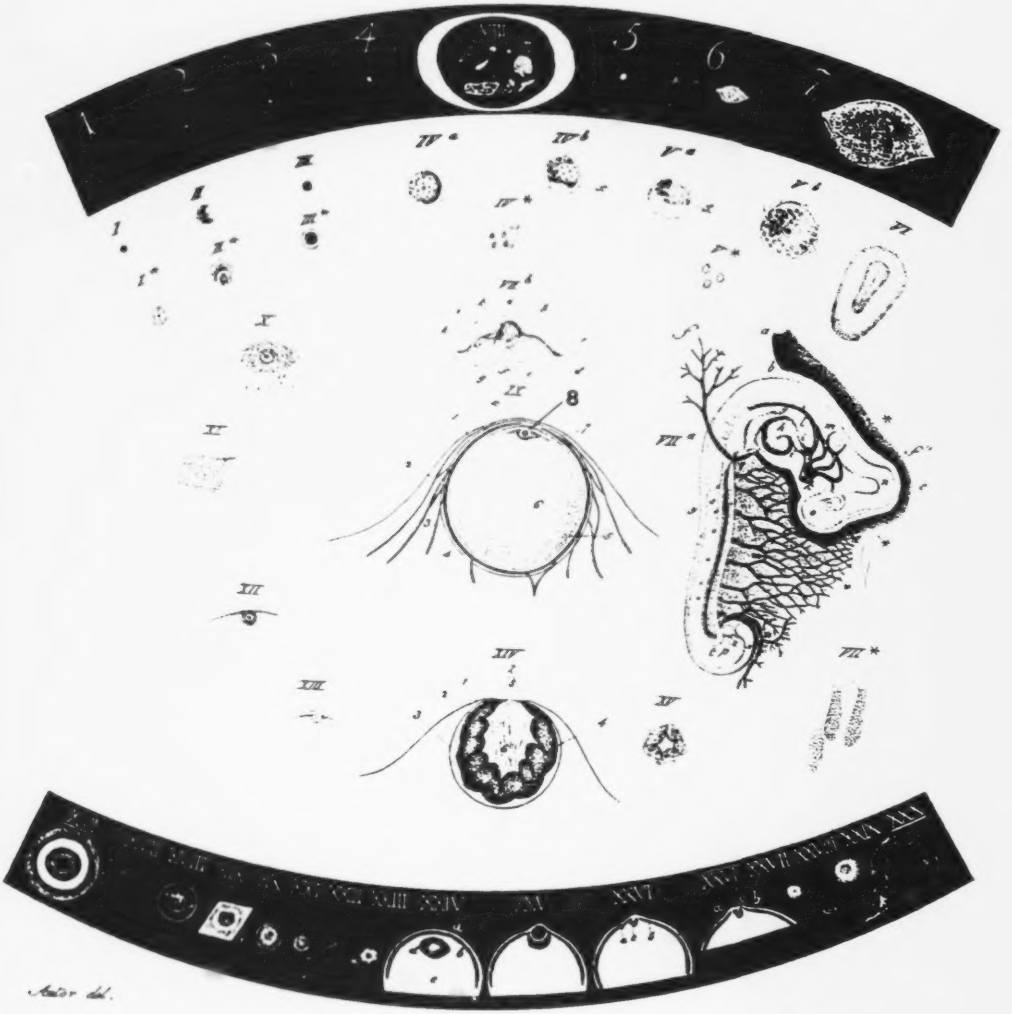


FIG. 5. Von Baer's (1827) illustrations of the mammalian ovum, 8 (retouched) in his figure IX, and various embryos.

spinal cord, somites and other structures. Figure XIII shows the human ovum magnified ten times. It is of interest to note that von Baer's first observations were made on a dog belonging to the professor with whom von Baer was working, and that the dog was sacrificed for that specific purpose.

The 1827 paper closes with four main conclusions. It is interesting to list them because they show what a dominant mind of the time regarded as important.

Every animal which springs from the coition of male and female is developed from an ovum, and none from a simple, formative liquid.

The male semen acts through the membrane of the ovum, which is pervious by no foramen, and in the ovum it acts first on certain innate parts of the ovum.

All development proceeds from the center to the periphery. Therefore the cen-

> tral parts are formed before the peripheral.
>
> The same method of development occurs in all vertebrate animals, beginning at the spine.

Note in the first conclusion the statement "every animal." Neither von Baer nor all the biologists since him have studied all animals. The important methodological principle here is that scientists assume that there are general rules that apply to natural phenomena—all is not chaos—and that one need but sample to find rules with broad applicability. This broad principle was not von Baer's. Harvey had made such a statement two centuries before.

The second conclusion is vague to the point of being meaningless yet, in the decades to come, fundamental advances in cytology, genetics, and embryology would emerge from studies on the interactions of sperm and ova.

The third conclusion is essentially correct and it was to be explained satisfactorily with the experiments of the Spemann school in the 20th century.

In the final conclusion von Baer agrees with many students of development beginning with Aristotle. A few decades later, observations of this sort, linked to the theory of evolution, were to change the way embryos were studied and to mold the conclusions reached.

VON BAER'S ACHIEVEMENTS

When we consider the achievements of von Baer we do not accept what he considered as important conclusions, as just listed, but emphasize his work in relation to later discoveries. The most notable was, of course, his detailed and abundantly confirmed description of the origin of the mammalian ovum from the Graafian follicle and its movement down the oviduct to the uterus, developing *en route*, was convincing.

> It is thus clear that v. Baer's great discovery did not come out of the blue sky,—no discovery ever does. The young Königsberg anatomist was well acquainted with the works of his predecessors. None of these had actually clinched the argument, and the best proof of it is that they had failed to convince anybody; they had not even completed their own conviction. Yet, thanks to their efforts the concept of mammalian egg had become plausible; it had been rescued from the oblivion to which Haller's absolutism had condemned it. Prejudices were not yet overcome, but the question was reopened and it was now possible for an intelligent and persistent man of science to investigate it anew, more thoroughly, and perhaps to solve it. This was v. Baer's achievement (Sarton, 1931, p. 319).

A second major discovery, often credited to von Baer, is reflected in his Figure VII^b, a cross section of a dog embryo magnified ten times. One can recognize embryonic layers. This conceptual advance was first made in 1817 by his colleague Pander. According to Oppenheimer (1967, p. 296),

> It is fully acknowledged that by demonstrating in terms of Pander's germ layers the true meaning of Wolff's concept of epigenesis, [von Baer] transformed embryology into a systematic and comparative science.

The concept was greatly elaborated by von Baer (1828), who extended it to include all major vertebrate embryos, and it gradually took on its classical form, that is, that embryos pass through a stage when they seem to be composed of three layers now known as ectoderm, mesoderm, and endoderm. The entire structure of the later embryo and adult is derived from these three layers.

Figure VII^b also shows the general features of a vertebrate embryo. The neural tube appears as a circle at top center. The tiny structure immediately below is the notochord, which von Baer discovered. He mentions that it is thinner than a hair and, in so doing, uses a scale unfamiliar to modern-day embryologists—we think of notochords only as they appear under the microscope where they seem to be about the same diameter as a half inch dowel. He describes the notochord as

> a streak which runs exactly in the axis of

> the future vertebral column and, therefore, of the entire fetus . . . . Because it is so slender, it can be recognized when it is first formed only if the water in which the embryo is being investigated is very free of yolk spherules . . . . This chord is obviously identical with the cartilaginous column found throughout life in the vertebral column of some cartilaginous fishes . . . . The notochord is not only the axis around which the first parts of the fetus form, but also the true measuring rod for the whole body and all the principal systems (quoted from Adelmann, 1966, pp. 1195–1196).

Von Baer did not get the relationship of notochord and vertebral column quite right—later it was found that the notochord is replaced by the vertebrae, not transformed into them.

Von Baer had a considerable interest in the general principles of development. He maintained, even as Aristotle had, that development in all vertebrates was essentially the same. He also maintained that early embryos are generalized and only later do they become specialized.

> *The more special develops from a more general type.* The development of the chick bears witness to this at every moment. In the beginning, when the back closes [*i.e.*, neural folds close], it is a vertebrate, and nothing more. When it constricts itself off from the yolk, and its gill clefts close and the allantois forms, it proves itself to be a vertebrate that cannot live in the water. Then later the two intestinal caeca form, a difference appears in the extremities, and the beak begins to appear; the lungs push upward, the rudiments of the airsacs are apparent, and we no longer can doubt that we are looking at a bird. While the character of the bird becomes still more evident through further development of the wings and airsacs, through fusion of the carpals, and so forth, the web between the toes vanishes and we recognize a land bird. The beak and feet proceed from a general shape to a particular one, the crop develops, the stomach has already divided into two chambers, the nasal shield appears. The bird attains the character of a gallinaceous bird, and finally, that of a domestic chicken (quoted by Oppenheimer, 1963, pp. 12–13).

Statements such as this were used by some in support of a rigid theory of recapitulation, which von Baer himself opposed.

Jane Oppenheimer (1963), in her fine study of von Baer, states that his "great contribution to embryology was a vast synthetic scheme," as shown by the following statement of von Baer:

> each step forward in development is made possible only by the preceding state of the embryo, nevertheless the total development is governed and directed by the whole essence of the animal that-is-to-be. And thus conditions at any moment are not alone absolutely determining for the future (p. 18).

But Oppenheimer points out that much of his "vast synthetic scheme" is what *we* read into von Baer. She offers this important caution, which we should always remember when the past is studied for its anticipations of the present:

> Although von Baer did comment, as we have seen, on sequences of events and conditions of the moment, he did not organize or arrange his remarks on them. When the excerpts quoted above are assembled out of context, as here, and placed in order, they suggest that von Baer had intimations of many important later concepts. But the reader must be warned to remember that such remarks are rare in the long volumes, not collected into a single chapter, and none were mentioned by von Baer in his summaries; they seem to have the quality of *obiter dicta* (p. 18).

Do we create the Fathers of Embryology in our own image?

Today von Baer is remembered primarily for two important discoveries and, as is so frequent in science, he was neither first nor entirely right in either.

One was the true egg of mammals. But, in fact an Englishman, William Cruikshank

(1797), had observed eggs in the oviduct of rabbits three days after mating. In addition, in 1824 Prévost and Dumas had published similar observations of an egg in the oviduct. Von Baer was aware of these anticipations but it was he who worked out some of the details of the relation of the Graafian follicles and eggs. He had erred, as noted before, in regarding the Graafian follicle as an egg and what we now know to be the true egg as "the fetal egg within the maternal egg."

His second main contribution has to do with the germ layers. Although Pander had first developed this concept, von Baer greatly extended it. He differed from later workers in recognizing four layers, counting the mesoderm as two because it splits later in development.

Nevertheless the accomplishments of von Baer were impressive and we are justified in ranking him, for his time, as the greatest embryologist since Aristotle. The fact that he could be so outstanding depended not only on his innate strengths but also on his opportunity to build on the publications and techniques of his predecessors ("He stood on the shoulders of Titans"). Science is a strongly accretive discipline and even a moderately gifted scientist can soon surpass the level of understanding and technical abilities of more illustrious predecessors. That much is expected.

But von Baer really did make a quantum leap. His monographs were far superior in detail and accuracy (but not in illustrations) to any work in embryology before his time. He was not overly concerned with theory but he was a vigorous opponent of the then currently exaggerated concept of recapitulation. His extensive knowledge of embryos led him to emphasize these points.

1. The embryos of different species belonging to a major taxonomic group resemble each other more closely early in development than they do as older embryos.

2. The embryos of higher species are like the embryos of lower species but not like the adults of lower species.

3. Thus if one compares the course of development of embryos of different taxonomic groups they are found to diverge progressively and not recapitulate different levels of organization.

Mayr (1982, pp. 469–479) has a fine discussion of this entire question. Remember that von Baer and others who speculated about embryos recapitulating a *scala naturae* did so before 1859 and Darwin's *On the Origin of Species.*

NINETEENTH CENTURY BIOLOGY: FROM UNITY TO FRAGMENTATION

One of the more striking aspects of early 19th century biology was its unified approach to problems. We have spoken of Harvey, Malpighi, von Baer and many others as "embryologists," but they were far more than that. Those general biologists, or naturalists in those days, did not approach the phenomena of life as geneticists, evolutionary biologists, cell biologists, or developmental biologists. Those disciplines, so distinct today, were then part of a conceptual whole. This unity came not from the recognition of fundamental principles but from the general lack of such principles. Those who studied embryos were interested in the material contributions of parents to offspring (cytology), what might be the "information" transferred (genetics), how the course of development related to the *scala naturae* (evolutionary biology), as well as the details of development itself (developmental biology).

Developmental biology was the most advanced and distinct of these four disciplines. The others gradually separated from the core of general biology. In the 1830s cytology was beginning to become a distinctive discipline. In the 1850s evolutionary biology would follow and, finally, genetics would become more independent and active in 1900.

There is a parallelism between some of von Baer's conclusions for different sorts of embryos, such as 1 and 3 just listed, and the different sorts of biologies. Both embryos and the biologies were much alike in their early stages and, as information was translated in the former and obtained for the latter, there was divergence among the embryos and among the branches of biology. Thus the embryos of the vertebrate classes are alike when very young but become very different as adults. In the same way, genetics, cytology, evolutionary biology, and embryology all deal with the same grand problems of life and continuity. We observe an excessive fragmentation of biology today but it is not unreasonable to predict that by the year 2000, or hopefully earlier, the extraordinary advances in the discrete branches of biology will be the basis for a new and far more satisfying general synthesis.

THE CELLS OF EMBRYOS

Those aspects of cytology that are important to genetics were discussed in some detail in last year's essay (III, pp. 607–639, 653–664, 687–721). Much of that is also relevant to embryology but only the main points will be repeated here.

First, of course, was the realization that the bodies of embryos, as well as adults, are composed solely of cells or cell products. Cells were first described in a number of plants by Robert Hooke in 1665. As more and more plants were studied, it began to look as though a basic principle was emerging—the bodies of *all* plants are composed of cells or cell products. Cell walls were assumed to be restricted to plants. No such structures appeared to be present in animals but slowly it came to be realized that animal tissues did have some structures that were present also in plant cells. That is, apart from walls, one could recognize similarities. This point of view was pressed especially by Theodor Schwann (1839) who made a fundamental change in definition. He suggested that cells should be defined on the basis of containing a nucleus rather than on being surrounded by walls. That change made it possible to recognize cells as the basic units of structure of both animals and plants. Embryos proved to be excellent material for detecting cells and many of Schwann's illustrations were of them (III, p. 613, fig. 4).

The next landmark hypothesis was that advanced in 1859 by Virchow (1863). He suggested that cells do not arise *de novo*, as Schwann had believed, but only by division of preexisting cells (*omnis cellula e cellula*). Knowledge of cells continued to increase but later developments, especially those analyzing the nucleus, will be considered when we reach Wilhelm Roux.

TRUE BEYOND ALL REASONABLE DOUBT?

A goal of science is to be able to say that some statement about a natural phenomenon is "true beyond all reasonable doubt." Such statements become parts of a broad theory that both synthesizes available information and suggests new observations to make and experiments to do that will extend the theory.

Care must be taken to ensure that students understand the difference between statements that are already accepted as true beyond all reasonable doubt and those hypotheses that, no matter how reasonable, have yet to be extensively tested before they can be accepted as true beyond all reasonable doubt. Years, decades, centuries, or even millennia may intervene between these two positions. For example, students are sometimes left with the impression that Schwann and Virchow made discoveries that were immediately accepted by biologists. Not at all. Both were proposing hypotheses that were accepted as useful ways of looking at the microscopic structure of organisms only after very extensive observation and experimentation on many kinds of plants and animals. In fact, neither hypothesis was capable of absolute proof. Absolute proof would require that the bodies of all individuals of all species of organisms must be checked for their cellular nature and for the origin of those cells.

Such exhaustive tests are not required in biology any more than in chemistry. Chemists are not required to study all water in order to say that it is composed solely of hydrogen and oxygen in certain proportions. Scientists are required to do no more than study an adequate sample. Nature behaves as though there is uniformity in materials and processes and, once the limits of variation have been ascertained, repetition of observation and experimentation is pointless. Not for all time, of course. When better ways of collecting data become available, repetition is required. Statements about animal structure gained with the unaided eye had to be checked first with simple lenses, then with compound microscopes, and now with electron microscopes.

As more and more organisms were studied, the hypotheses of Schwann and Virchow seemed to be true in most instances—but not in all. In some situations, such as the early embryos of insects and stages in the life cycle of slime molds, the body consists of nuclei in a cytoplasm not divided into discrete cells—a syncytium. As for the origin of cells, reports continued even into the 20th century of *de novo* formation but these have been shown to have been errors.

Thus there is a certain amount of wobble even in those biological statements that are the foundations of the science. This is not of any great concern since biologists have come to see that the phenomena of life can be described as themes with variations.

In the early 19th century there were very few statements about embryos that could be accepted as true beyond all doubt (excluding some exceptions). Aristotle in the 4th century B.C. and Harvey in the 17th century A.D. both believed that development was epigenetic but they only proposed, they did not prove. Acceptable proof came slowly following the observations of Wolff.

In fact, about all that could be said as true beyond all reasonable doubt in von Baer's time, the early 19th century, was:

1. Development is epigenetic, that is, there is differentiation with growth.
2. But there must be some sort of preformation that will account for the transfer of information from the parental to the filial generation.
3. There is considerable resemblance in the embryonic development of species within the same taxonomic group, the resemblances being greater in younger stages than in later stages.
4. The bodies of early embryos seem to be composed of layers from which the structures of the larva and adult are derived.

In addition to these few concepts, there was a rapidly increasing body of information on the normal development of a wide variety of organisms. This body of information was about to be incorporated into the most basic biological concept—organic evolution.

THE DEVELOPMENT OF CHARLES DARWIN

The paradigm shift of 1859 changed not only what biologists did but also provided a rationale for their research. The new paradigm was also able to offer a more satisfying explanation for much that had already been learned. In fact, the data themselves seemed to be awaiting some organizing theory and a simple idea provided it. But ideas are not simple until after they have emerged. Nevertheless many biologists must have wondered as did Thomas Henry Huxley (1888, p. 197):

> My reflection, when I first made myself master of the central idea of the "Origin" was, "How extremely stupid not to have thought of that!"

Darwinism provided a new way of thinking about a cluster of major biological phenomena related to embryology:

1. Living organisms seemed to form a continuum from the least complex to human beings—a Great Scale of Being or the *scala naturae*.
2. Embryos of species in the same taxonomic group resemble one another.
3. Recapitulation.
4. Homology.

Each of these phenomena was so striking and so pervasive that it was impossible not to think that there must be some underlying cause. Today we see all of this as a reflection of evolutionary change but that clarity of vision started only in 1859.

The recognition of types of organisms, or major groupings, must have been part of the way human beings looked upon the natural world from the very earliest of times. There were animals with hair, the mammals; animals with feathers, the birds; animals with scales that lived on land, the reptiles; animals generally with a smooth moist skin, the frogs; and animals with scales that lived in the water, the fishes. These were discrete groups without a closely graded series of intermediate forms. One could even lump all of these in a still grander group—animals with a backbone and many other similarities. Organisms sharing similar features could be classified, that is, placed in taxonomic groups. What could be the basis of these groupings?

Although the taxonomic groups were discrete, nevertheless it was possible to arrange them in a scale of complexity and similarity. This "*scala naturae*," or "chain of being" extended not only from human beings to the simplest organisms—protozoans and bacteria discovered by the early microscopists—but to lifeless matter as well. The *scala naturae* was a theological construct describing the immutable forms of life and non-life that had been created. It could be regarded as a ladder that found the least complex objects on the bottom rung and human beings at the top—just below the divine itself (Lovejoy, 1936; Eiseley, 1858; Ritterbush, 1964; Gould, 1977; Mayr, 1982).

Groups of organisms not only shared features when they were adults but Aristotle and all others after him realized that embryos were also alike in many ways. An equally important discovery was that the similarities were greater in younger than in older embryos.

By the early 1800s it seemed to some that there was a parallel between the *scala naturae* and the course of embryonic development and this led to the hypothesis of recapitulation. In its extreme form this meant that the embryos of higher forms (mammals for example) went through stages that resembled the *adults* of lower forms (fish, amphibians).

And finally there was the concept of homology. During the 18th century there had been a great increase in knowledge of the structure of organisms and some tantalizing phenomena were discovered. For example, it was found that the limbs of tetrapods seemed to be built on the same general plan. The arms seemed to have a basic plan of one proximal bone and two more distal; the hand had several bones forming a wrist, about five in the palm, and finally a few in each finger. Even the wings of birds and bats could be understood to be variations on this basic plan. The corresponding bones were said to be "homologous." That is, the humerus is really the "same thing" in different species.

It was recognized that homology is based on more than superficial resemblances. The wings of insects, for example, do not share the same basic structure. Wings of birds (or bats) and insects were said to be "analogous." Analogy, then, was restricted to functionally similar, but morphologically different, structures. Homology was restricted to morphologically similar structures that might be functionally similar (appendages of horses and frogs) or not (appendages of porpoise, bat, and monkeys).

And Darwin put it all together. In Chapter 13 of the *Origin* he sought to

> explain these several facts in embryology, [1] namely the very general, but not universal differences in structure between the embryo and the adult; [2] of parts in the same individual embryo, which ultimately become very unlike and serve for diverse purposes, being at this early period of growth alike; [3] of embryos of different species within the same class, generally, but not universally, resembling each other; [4] of the structure of the embryo not being closely related to its conditions of existence, except when the embryo becomes at any period of life active and has to provide for itself; [5] of the embryo apparently having sometimes a higher organisation than the mature animal, into which it is developed (pp. 442–443; I added the numbers in brackets).

The explanation of these five phenomena, which we hardly recognize as problems today, was not obvious in Darwin's time.

> There is no obvious reason why, for instance, the wing of a bat, or the fin of a porpoise, should not have been sketched out with all the parts in proper proportion, as soon as any structure became visible in the embryo (p. 442).

Yet when they first start to form in the embryo the wing and fin are nearly the same. They diverge later.

Darwin thought that this similarity of fin and wing and the other problems he listed could be explained on the basis of three assumptions: first, evolution; second, that the modifications that occur in the course of evolution "may have supervened at a not very early period in life" (that is late in life); and third, "that at whatever age any variation first appears in the parent, it tends to reappear at a corresponding age in the offspring" (p. 444).

> Now let us apply these facts and the above two [he did not list evolution but it was implied] principles—which latter, though not proved true, can be shown to be in some degree probable—to species in a state of nature. Let us take a genus of birds, descended on my theory from some one-parent species, and of which the several new species have become modified through natural selection in accordance with their diverse habits. Then, from the many slight successive steps in variation having supervened at a rather late age, and having been inherited at a corresponding age, the young of the new species of our supposed genus will manifestly tend to resemble each other much more closely than do the adults . . . . We may extend this view to whole families or even classes. The fore-limbs, for instance, which served as legs in the parent-species, may become, by a long course of modification, adapted in one descendant to act as hands, in another as paddles, in another as wings; and on the above two principles—namely of each successive modification supervening at a rather late age, and being inherited at a corresponding late age—the fore-limbs in the embryos of the several descendants of the parent-species will still resemble each other closely, for they will not have been modified (pp. 446–447).

This line of reasoning can explain facts 1, 2, and 3, listed before—problems that Darwin puzzled about. Fact 4 refers to the lack of relation of embryos to their environment except when they are *active*. Active embryos are those that become free-living and secure their own food—in contrast with those of birds, for example, which have all the food they need stored in the egg. Many species of invertebrates have small eggs, with very little yolk. These eggs develop into free-living larvae that obtain their own food after a very short period. Many of these are microscopic, ciliated creatures having no resemblance to the adults they will become. Darwin looked upon them as having evolved the ability to develop quickly into a food-getting stage.

Darwin's fifth fact in need of explanation is that some embryos appear to be more complex than the adults. This made sense with an hypothesis of evolution but not otherwise. Many parasitic organisms, for example, have embryonic stages that are as complex as those of related free-living species but these develop into degenerate adults. It was difficult to explain why embryos of these parasites developed structures that later become reduced or lost unless it is assumed that the embryos are retaining the fundamental patterns of development characteristic of their taxonomic group.

Darwin's hypothesis of descent with modification, natural selection acting on the hereditary differences among individuals of a species, did far more than make some otherwise confusing embryological phenomena understandable. It accounts for the grand phenomenon of organisms belonging to sets or taxonomic groups.

> As all the organic beings, extinct and recent, which have ever lived on this earth have to be classed together, and as all have been connected by the finest gradations, the best, or indeed, if our collections were nearly perfect, the only possible arrangement, would be genealogical. Descent being on my view the hidden bond of connections which naturalists have been seeking under the term of the natural system. On this view we can understand how it is that, in the eyes of most naturalists, the structure of the embryo is even more important for classification than that of the adult. For the embryo is the animal in its less modified state; and in so far it reveals the structure of its progenitor. In two groups of animals, however much they may at present differ from each other in structure and habits, if they pass through the same or

> similar embryonic stages, we may feel assured that they have both descended from the same or nearly similar parents, and are therefore in that degree closely related. Thus, community in embryonic structure reveals community of descent. It will reveal this community of descent, however much the structure of the adult may have been modified and obscured; we have seen, for instance, that cirripedes [barnacles] can at once be recognized by their larvae as belonging to the great class of crustaceans. As the embryonic state of each species and group of species partially shows us the structure of their less modified ancient progenitors, we can clearly see why ancient and extinct forms of life should resemble the embryos of their descendants,—our existing species (pp. 448–449).

Here Darwin was applying his concept of evolution to the widely accepted but poorly understood hypothesis of recapitulation. But he was cautious in accepting recapitulation without reservation. He continues,

> Agassiz believes this to be a law of nature; but I am bound to confess that I only hope to see the law hereafter proved true. It can be proved true in those cases alone in which the ancient state, now supposed to be represented in many embryos, has not been obliterated, either by the successive variations in a long course of modifications having been supervened at a very early age, or by the variations having been inherited at an earlier period than that at which they first appeared. It should also be borne in mind, that the supposed law of resemblance of ancient forms of life to the embryonic stages of recent forms, may be true, but yet, owing to the geological record not extending far enough back in time, may remain for a long period, or for ever, incapable of demonstration.
>
> Thus, as it seems to me, the leading facts in embryology . . . are second to none in natural history . . . . Embryology rises greatly in interest, when we thus look at the embryo as a picture, more or less obscured, of the common parent-form of each great class of animals (pp. 449–450).

Darwin was so impressed with the data of embryology, together with that of morphology and classification (also discussed in his Chapter 13), that he concludes:

> Finally, the several classes of facts which have been considered in this chapter, seem to me to proclaim so plainly, that the inumerable species, genera, and families of organic beings, with which this world is peopled, have all descended, each within its own class or group, from common parents, and have all been modified in the course of descent, that I should without hesitation adopt this view, even if it were unsupported by other facts or arguments (pp. 457–458).

Darwin gave embryologists a mission of first-rate theoretical importance—the search for lineages in the minutiae of development. To be sure, embryos could do no more than reflect these lineages but, when fossil evidence was so meager, there was no alternative.

There had already been many triumphs that were known to Darwin. Barnacles were of special interest to him since he had produced the definitive monographs on these creatures. Barnacles are sessile animals enclosed in a shell. They resemble mollusks more than any other group of invertebrates. Cuvier, the most respected naturalist of the early 19th century, had included the barnacles in the Mollusca. It had been discovered, however, that the barnacle embryos develop into a larval type characteristic of the crustaceans. Darwin notes (p. 440),

> Even the illustrious Cuvier did not perceive that a barnacle was, as it certainly is, a crustacean; but a glance at the larva shows this to be the case in an unmistakable manner. So again the two main divisions of cirripedes, the pedunculated and sessile, which differ widely in external appearance, have larvae in all their several stages barely distinguishable.

Among the vertebrates there were many

examples known to Darwin of embryos apparently recapitulating stages found in putative ancestors. Birds and mammals, with only a single aorta in the adult, have in the embryo the six pairs characteristic of fishes (I, pp. 499–501). Bird and mammalian embryos develop in succession a pronephros, mesonephros, the kidneys of the lower vertebrates, and finally their own adult metanephric kidney (I, pp. 503–504). Possibly the most dramatic example is that of the development of the malleus, incus, and stapes of the mammalian ear from the jaw bones of lower forms (I, pp. 498–499). This was predicted on the basis of careful embryological work, as being highly probable, long before paleontologists unearthed the mammal-like reptiles that provided absolute proof.

Thus by Darwin's time embryology had come to be more than the detailed study of successive stages in the development of organisms. The data of descriptive embryology could be used to suggest the course of evolution and to classify organisms into natural groups.

Homology was also clarified by embryology. The meaning of the "same thing" could now be understood. A common structure in an ancestor would change in the course of the evolution of different daughter species. The structure would still be the "same thing," although variously modified. In the case of hard structures, such as bones, it might be possible to trace the changes in fossils of different ages (jaw bones and ear ossicles, for example). Such would not be possible for soft parts (vertebrate kidneys and aortic arches) but often the embryos provided a clue. Homology, then, was defined as identity of embryonic origin.

RECAPITULATION

In the decades after Darwin, the fundamental theorem of evolutionary embryology was recapitulation. This concept was formulated well before Darwin and it expressed the relationship of embryogenesis to classification and to the *scala naturae.* When the Darwinian paradigm reinterpreted the *scala naturae* as the consequence of descent with modification, the data of embryology were reinterpreted as well.

The concept of recapitulation has had an incredible history and that history tells us nearly as much about the workings of science as about embryos. The concept occupied the center of theoretical embryology throughout the 19th century until it was displaced by the problem of the causes of differentiation. As Darwin suggested, many facts about development are inexplicable without the concept of recapitulation.

LOUIS AGASSIZ

It is fascinating to note how the concept of recapitulation, which itself suggests evolution, was used before the publication of *On the Origin of Species.* The case of Louis Agassiz (1807–1873) is especially interesting. He was a Swiss naturalist of great ability who spent much of his life in the United States. His famous *An Essay on Classification* was first published in 1857 as part of his *Contributions to the Natural History of the United States.* The *Essay* was republished in 1859—the year of the *Origin.*

It was well known in Agassiz's time that there is a general relation between the complexity of organisms and the time they first appear in the fossil record. This was a relationship that Darwin was to explain by his theory of evolution. Agassiz was never an evolutionist and he interpreted this relationship as an aspect of Creation. He described an especially striking example of the parallelism of complexity of living species and their fossil record in higher plants where

> we at once see how the vegetable kingdom has been successively introduced upon earth, in an order which coincides with the relative position its primary divisions bear to one another, in respect to their rank, as determined by the complication of their structure . . . . If the vegetable kingdom constitutes one graduated series [part of the *scala naturae*], beginning with the Cryptogams, followed by the Gymnosperms, and ending with the Monocotyledones and Dicotyledones, have we not in that series the

> most striking coincidence with the order of succession, as exhibited by the Cryptogams of the oldest geological formations, especially the Ferns, Equisetaceae, and Lycopodiaceae of the Carboniferous period, followed by the Gymnosperms of the Trias and Jura and the Monocotyledones of the same formation and the late development of the Dicotyledones? Here, as everywhere, there is but one order, one plan in nature (1859, p. 168).

There follows Section XXV, which is entitled "Parallelism between the geological succession of animals and the embryonic growth of their living representatives." Agassiz writes that

> Several authors have alluded to the resemblance which exists between the young of some of the animals now living and the fossil representatives of the same families in earlier periods (pp. 168–169).

These tended to be isolated cases yet Agassiz provided a number of examples from among the invertebrates and this conclusion is reached:

> It may therefore be considered as a general fact, very likely to be more fully illustrated as investigations cover a wider ground, that the phases of development of all living animals corresponds to the order of succession of their extinct representatives in past geological times. As far as this goes, the oldest representatives of every class may then be considered as embryonic types of their respective orders or families among the living (p. 174).

And the cause?

> It exhibits everywhere the working of the same creative Mind, through all times, and upon the whole surface of the globe (p. 175).

Thus in pre-Darwinian days it was recognized that the positions of organisms in the *scala naturae* might be parallel to the times of their first appearance in the fossil record, and to their patterns of development.

ERNST HAECKEL

Recapitulation became a truly baroque edifice in the hands of Ernst Heinrich Phillip Haeckel (1834–1919), a dominant personality in German science of the last half of the 19th century. The concept was eventually regarded as either wrong or useless, yet it remains true today that some extraordinary phenomena "make sense" on the basis of a more balanced statement of the concept. The rejection of the concept of recapitulation in the late 19th and early 20th centuries is probably a case, as Gould states (1977, p. 2), of "throwing the baby out with the bath water." I agree.

Haeckel's theory was proposed in his *Generelle Morphologie* of 1866, and revised in *Naturliche Schöpfungschichte* (1868; trans. 1876), and in *Anthropogenie* (1874; trans. 1905*a*, 1905*b*).

If the concept of recapitulation is accepted as a useful way of looking at some otherwise puzzling embryological phenomena rather than as a fundamental and relentless Law of Nature, it becomes a powerful heuristic device. But such a view demands that we modify Haeckel's striking formulation, "ontogeny recapitulates phylogeny," by adding "not quite" and "sometimes."

Haeckel did far more than formulate his aphorism—he attempted to provide a conceptual scheme for all of descriptive embryology. He provided an overall theory that described the history of the development of individuals and which paralleled Darwin's theory that described the history of the development of species. Furthermore he suggested a close relationship between the two.

> These two branches of our science—on the one side ontogeny or embryology, and on the other phylogeny, or the science of race-evolution—are most vitally connected. The one cannot be understood without the other. It is only when the two branches fully co-operate and supplement each other that "Biogeny" (or the science of the genesis of life in the widest sense) attains the rank of a philosophic science. The connection

> between them is not external and superficial, but profound, intrinsic, and causal. This is a discovery made by recent research, and it is most clearly expressed in the comprehensive law which I have called "the fundamental law of organic evolution," or "the fundamental law of biogeny." This general law, to which we find ourselves constantly recurring, and on the recognition of which depends one's whole insight into the story of evolution, may be briefly expressed in the phrase: "The history of the foetus is a recapitulation of the history of the race"; or, in other words, "Ontogeny is a recapitulation of phylogeny." It may be more fully stated as follows: The series of forms through which the individual organism passes during its development from the ovum to the complete bodily structure is a brief, condensed repetition of the long series of forms which the animal ancestors of the said organism, or the ancestral forms of the species, have passed through from the earliest period of organic life down to the present day . . . .
>
> Phylogenesis is the mechanical cause of ontogenesis. In other words, the development of the stem, or race, is, in accordance with the laws of heredity and adaptation, the cause of all the changes which appear in a condensed form in the evolution of the foetus.
>
> The chain of manifold animal forms which represent the ancestry of each higher organism, or even of man, according to the theory of descent, always forms a connected whole. We may designate this uninterrupted series of forms with the letters of the alphabet: A, B, C, D, E, etc., to Z. In apparent contradiction to what I have said, the story of the development of the individual, or the ontogeny of most organisms, only offers the observer a part of these forms; so that the defective series of embryonic forms would run: A, B, D, F, H, K, M, etc.; or, in other cases, B, D, H, L, M, N, etc. . . . . In the embryonic succession much is wanting that certainly existed in the earlier ancestral succession. If the parallel of the two series were complete, and if this great fundamental law affirming the causal connection between ontogeny and phylogeny in the proper sense of the word were directly demonstrable, we should only have to determine, by means of the microscope and the dissecting knife, the series of forms through which the fertilized ovum passes in its development; we should have before us a complete picture of the remarkable series of forms which our animal ancestors have successively assumed from the dawn of organic life down to the appearance of man. But such a repetition of the ancestral history by the individual in its embryonic life is very rarely complete. We do not often find our full alphabet. In most cases the correspondence is very imperfect, being greatly distorted and falsified (1905*b*, pp. 2–3).

Part of the defect in the alphabet of descent is to be expected:

> In this evolutionary appreciation of the facts of embryology we must, of course, take particular care to distinguish sharply and clearly between the primitive, palingenetic (or ancestral) evolutionary processes and those due to cenogenesis. By *palingenetic* processes, or embryonic *recapitulations,* we understand all those phenomena in the development of the individual which are transmitted from one generation to another by heredity and which, on that account, allow us to draw direct inferences as to corresponding structures in the development of the species. On the other hand, we give the name of *cenogenetic* processes, or embryonic *variations,* to all those phenomena in the foetal development that cannot be traced to inheritance from earlier species, but are due to the adaptation of the foetus, or the infant-form, to certain conditions of its embryonic development (1905*b*, p. 4).

Haeckel, along with most evolutionists at the time, accepted both Darwinism and Lamarckism. The distinction he is making between palingenetic and cenogenetic sounds strange to modern ears but his examples show what he had in mind. He regards

> as *palingenetic* the formation of the two primary germinal layers and of the primitive gut, the undivided structure of the dorsal nerve-tube, the appearance of a simple axial rod [the notochord] between the medullary tube and gut, the temporary formation of the gill-clefts and arches, the primitive kidneys, and so on. All these, and many other important structures, have clearly been transmitted by a steady heredity from the earliest ancestors of the mammal, and are, therefore, direct indications of the presence of similar structures in the history of the stem.
>
> On the other hand, this is certainly not the case with the following embryonic forms, which we must describe as *cenogenetic* processes: the formation of the yelk-sac, the allantois, the placenta, the amnion, the serolemma, and the chorion (1905*b*, p. 4).

These structures listed as cenogenetic are characteristic of living mammals so, presumably, first evolved in them or in the mammal-like reptiles and, hence appeared late in phylogeny.

In the 1860s when Haeckel began to speculate about the phylogeny leading to the human species, the fossil record was mainly gaps (as it still is), accurate knowledge of chromosomal cytology, genetics and biochemistry was essentially nil, microscopists knew little about the biology of what we now call the prokaryotes, and even the mechanisms of evolutionary change were poorly understood. The most valuable biological information was to be found in comparative morphology and embryology. These two disciplines, therefore, provided the evidential basis for Haeckel's version of recapitulation.

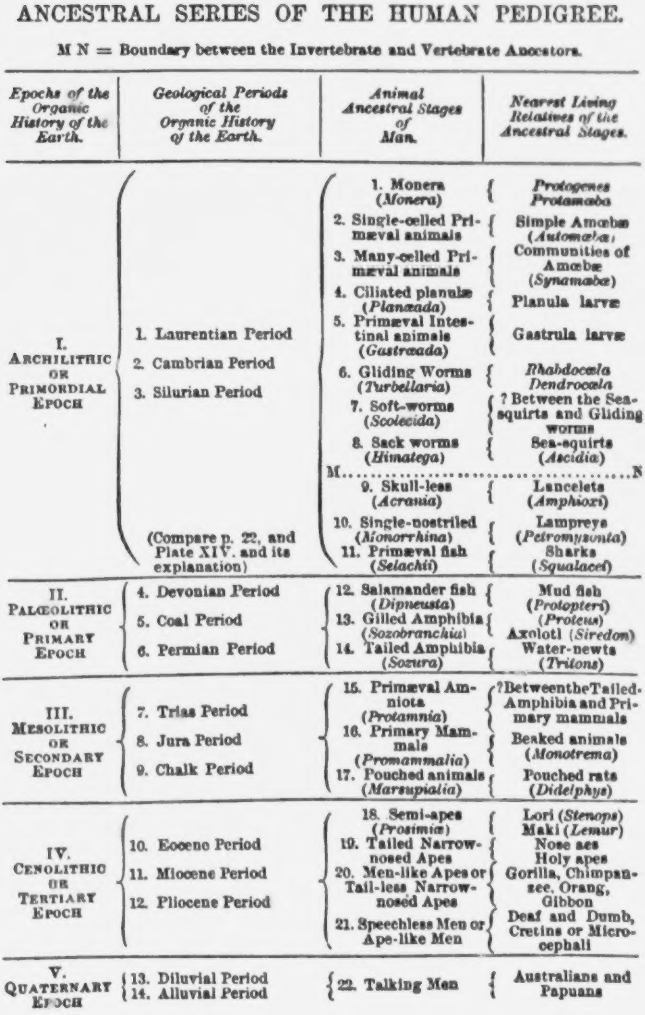
One of Haeckel's early attempts (1876, vol. 2, p. 295) to imagine what the phylogeny from primitive monad to human being might be is shown here as Figure 6. Twenty-two major steps from Monera to Talking Man are recognized. The columns to the left of the ancestral stages show the geological time that those stages were thought to have first appeared, beginning with the Monera in the Laurentian (or Precambrian). The rightmost column shows the nearest living species to the ancestral stages.

Later (Haeckel 1905*a*) increased these ancestral stages to 30 as shown in Figures 7 and 8. The three rightmost columns show the relative values of the data ranging from 0, !, or I in cases of no supporting evidence to three horizontal I's, !!!, or III for the best.

Again his starting point is a group known as the Monera, a name that remains to this day in some schemes of classifications (R. H. Whittaker, 1969) as the Kingdom of the prokaryotes and so includes the bacteria, blue-green algae (and sometimes the viruses). Very little was known about the nature of these organisms in Haeckel's time. To him they consisted of "simple, homogeneous, albuminous matter (protoplasm)." In fact they were the simplest organisms that one could imagine. They were thought to be entirely without nuclei or other structures. They were assumed to have first appeared in the very earliest of PreCambrian times by spontaneous generation. The starting materials were assumed to be simple combinations of carbon, oxygen, hydrogen, and nitrogen.

The next stage is that of single celled animals, such as amoeba. In Figures 9–12 Haeckel (1905*a*) shows a comparison of the putative ancestors and the events in human ontogeny. Thus the first phylogenetic stage corresponds to the undivided human fertilized ovum, although he believed that there might be a remnant of a Monera stage since for a while the egg nucleus seems to vanish (although not understood at the time the germinal vesicle does break down and "disappear").

Stage 5 of Figure 6, Stage 6 of Figure 7, and Stage 3 of Figure 9 all represent a very important ancestral type in Haeckel's scheme—the Gastraeades. Embryologists were familiar with the common pattern of



ANCESTRAL SERIES OF THE HUMAN PEDIGREE.

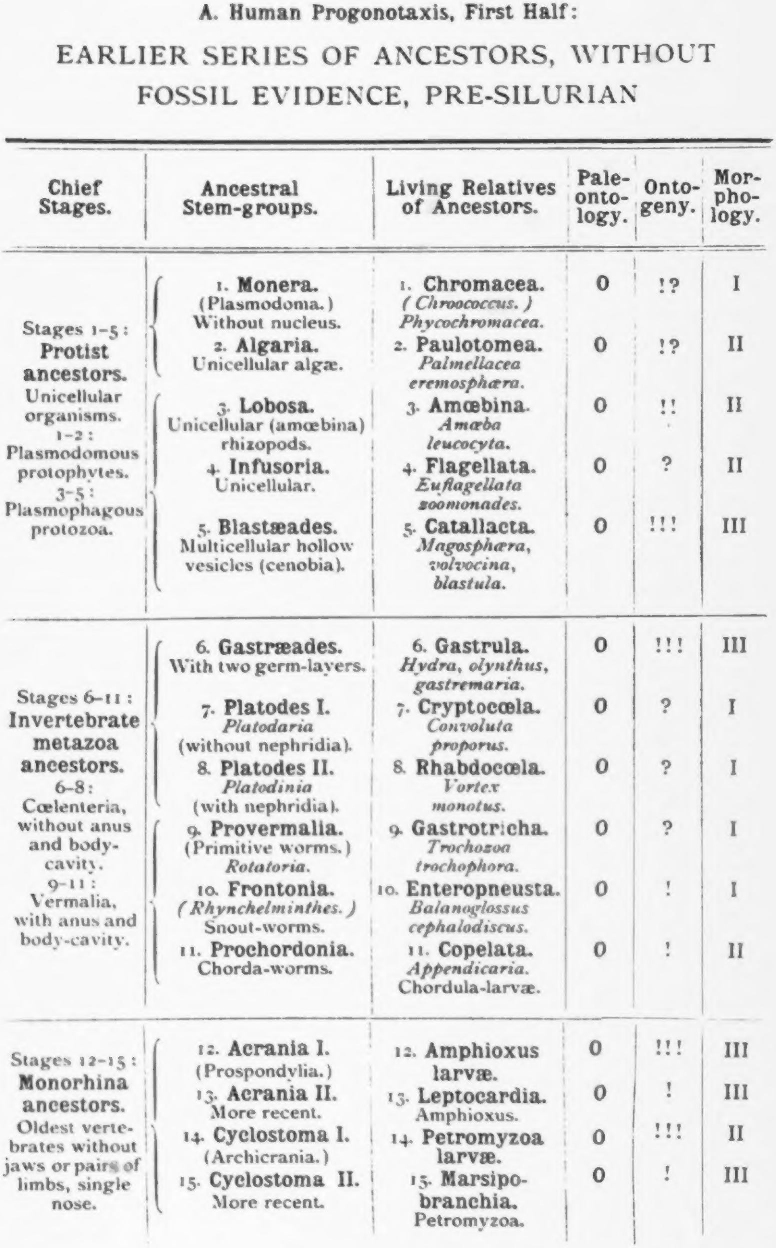
M N = Boundary between the Invertebrate and Vertebrate Ancestors.

Epochs of the Organic History of the Earth.	*Geological Periods of the Organic History of the Earth.*	*Animal Ancestral Stages of Man.*	*Nearest Living Relatives of the Ancestral Stages.*
I. ARCHILITHIC OR PRIMORDIAL EPOCH	1. Laurentian Period 2. Cambrian Period 3. Silurian Period	1. Monera (*Monera*)	*Protogenes* *Protamœba*
		2. Single-celled Primæval animals	Simple Amœbæ (*Autamœbæ*)
		3. Many-celled Primæval animals	Communities of Amœbæ (*Synamœbæ*)
		4. Ciliated planulæ (*Planæada*)	Planula larvæ
		5. Primæval Intestinal animals (*Gastræada*)	Gastrula larvæ
		6. Gliding Worms (*Turbellaria*)	*Rhabdocœla* *Dendrocœla*
		7. Soft-worms (*Scolecida*)	? Between the Sea-squirts and Gliding worms
		8. Sack worms (*Himatega*)	Sea-squirts (*Ascidiæ*)
M……………………………………………………N			
		9. Skull-less (*Acrania*)	Lancelets (*Amphioxi*)
		10. Single-nostriled (*Monorrhina*)	Lampreys (*Petromyzonta*)
	(Compare p. 22, and Plate XIV. and its explanation)	11. Primæval fish (*Selachii*)	Sharks (*Squalacei*)
II. PALÆOLITHIC OR PRIMARY EPOCH	4. Devonian Period 5. Coal Period 6. Permian Period	12. Salamander fish (*Dipneusta*)	Mud fish (*Protopteri*)
		13. Gilled Amphibia (*Sozobranchia*)	(*Proteus*) Axolotl (*Siredon*)
		14. Tailed Amphibia (*Sozura*)	Water-newts (*Tritons*)
III. MESOLITHIC OR SECONDARY EPOCH	7. Trias Period 8. Jura Period 9. Chalk Period	15. Primæval Amniota (*Protamnia*)	?Between the Tailed Amphibia and Primary mammals
		16. Primary Mammals (*Promammalia*)	Beaked animals (*Monotrema*)
		17. Pouched animals (*Marsupialia*)	Pouched rats (*Didelphys*)
IV. CENOLITHIC OR TERTIARY EPOCH	10. Eocene Period 11. Miocene Period 12. Pliocene Period	18. Semi-apes (*Prosimiæ*)	Lori (*Stenops*) Maki (*Lemur*)
		19. Tailed Narrow-nosed Apes	Nose aes Holy apes
		20. Men-like Apes or Tail-less Narrow-nosed Apes	Gorilla, Chimpanzee, Orang, Gibbon
		21. Speechless Men or Ape-like Men	Deaf and Dumb, Cretins or Microcephali
V. QUATERNARY EPOCH	13. Diluvial Period 14. Alluvial Period	22. Talking Men	Australians and Papuans

FIG. 6. Haeckel's correlations of geological age, putative human ancestors, and nearest living relatives (Haeckel, 1876, vol. 2, p. 295).

development in both invertebrates and vertebrates with eggs containing little yolk—the formation of a two-layered gastrula (Fig. 13). How was one to explain this seemingly fundamental pattern of development? Haeckel speculated that in very early geological times the ancestors of all metazoans had a body consisting of two layers enclosing a central cavity—the gut. This was his Gastraea Theory. Such creatures, the Gastraeades, were similar to the gastrula stage of the embryos of many living species and even to the adult stages of the most primitive living coelenterates.

Figure 14 shows the critical position of the Gastraeades in the phylogeny of the major groups of animals. Haeckel surmised the long evolutionary line to the mammals passed thereafter through worm-like stages and finally reached the chordates (Stage 8



A. Human Progonotaxis, First Half:

EARLIER SERIES OF ANCESTORS, WITHOUT FOSSIL EVIDENCE, PRE-SILURIAN

Chief Stages.	Ancestral Stem-groups.	Living Relatives of Ancestors.	Pale-onto-logy.	Onto-geny.	Mor-pho-logy.
Stages 1–5: **Protist ancestors.** Unicellular organisms. 1–2: Plasmodomous protophytes. 3–5: Plasmophagous protozoa.	1. **Monera.** (Plasmodoma.) Without nucleus.	1. **Chromacea.** *(Chroococcus.)* *Phycochromacea.*	0	!?	I
	2. **Algaria.** Unicellular algæ.	2. **Paulotomea.** *Palmellacea eremosphæra.*	0	!?	II
	3. **Lobosa.** Unicellular (amœbina) rhizopods.	3. **Amœbina.** *Amœba leucocyta.*	0	!!	II
	4. **Infusoria.** Unicellular.	4. **Flagellata.** *Euflagellata zoomonades.*	0	?	II
	5. **Blastæades.** Multicellular hollow vesicles (cenobia).	5. **Catallacta.** *Magosphæra, volvocina, blastula.*	0	!!!	III
Stages 6–11: **Invertebrate metazoa ancestors.** 6–8: Cœlenteria, without anus and body-cavity. 9–11: Vermalia, with anus and body-cavity.	6. **Gastræades.** With two germ-layers.	6. **Gastrula.** *Hydra, olynthus, gastremaria.*	0	!!!	III
	7. **Platodes I.** *Platodaria* (without nephridia).	7. **Cryptocœla.** *Convoluta proporus.*	0	?	I
	8. **Platodes II.** *Platodinia* (with nephridia).	8. **Rhabdocœla.** *Vortex monotus.*	0	?	I
	9. **Provermalia.** (Primitive worms.) *Rotatoria.*	9. **Gastrotricha.** *Trochozoa trochophora.*	0	?	I
	10. **Frontonia.** *(Rhynchelminthes.)* Snout-worms.	10. **Enteropneusta.** *Balanoglossus cephalodiscus.*	0	!	I
	11. **Prochordonia.** Chorda-worms.	11. **Copelata.** *Appendicaria.* Chordula-larvæ.	0	!	II
Stages 12–15: **Monorhina ancestors.** Oldest vertebrates without jaws or pairs of limbs, single nose.	12. **Acrania I.** (Prospondylia.)	12. **Amphioxus larvæ.**	0	!!!	III
	13. **Acrania II.** More recent.	13. **Leptocardia.** Amphioxus.	0	!	III
	14. **Cyclostoma I.** (Archicrania.)	14. **Petromyzoa larvæ.**	0	!!!	II
	15. **Cyclostoma II.** More recent.	15. **Marsipobranchia.** Petromyzoa.	0	!	III

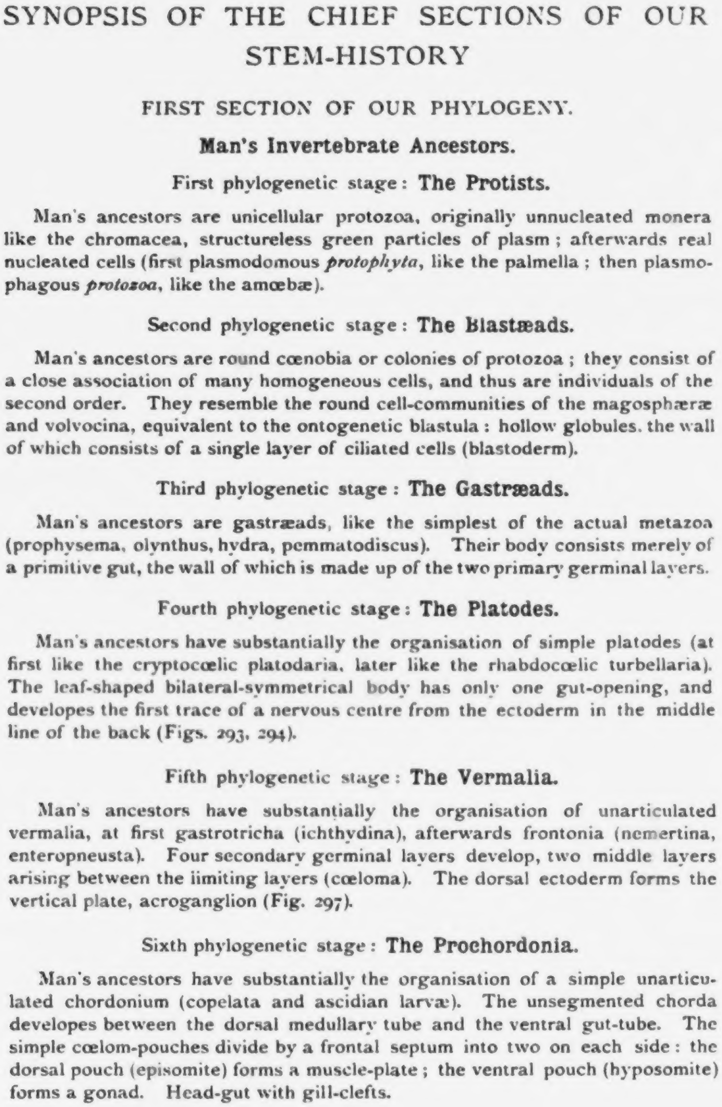
FIG. 7. The putative human ancestors among the invertebrates and lower vertebrates (Haeckel, 1905*a*, table 26).

B. Human Progonotaxis, Second Half:

LATER ANCESTORS, WITH FOSSIL EVIDENCE, BEGINNING IN SILURIAN PERIOD

Geological Periods.	Ancestral Stem-groups.	Living Relatives of Ancestors.	Pale-onto-logy.	Onto-geny.	Mor-pho-logy.
Silurian.	16. **Selachii.** Primitive fishes. *Proselachii.*	16. **Natidanides.** Chlamdoselachus. Heptanchus.	—	! !	III
Silurian.	17. **Ganoides.** Plated-fishes. *Proganoides.*	17. **Accipenserides.** (Sturgeons.) Polypterus.	=	!	II
Devonian.	18. **Dipneusta.** *Paladipneusta.*	18. **Neodipneusta.** Ceratodus. Protopterus.	—	! !	II
Carbonife-rous.	19. **Amphibia.** *Stegocephala.*	19. **Phanero-branchia.** Salamandrina. (Proteus, triton.)	≡	! ! !	III
Permian.	20. **Reptilia.** *Proreptilia.*	20. **Rhyncho-cephalia.** Primitive lizards. Hatteria.	≡	! !	II
Triassic.	21. **Monotrema.** *Promammalia.*	21. **Ornitho-delphia.** *Echidna.* *Ornithorhyncus.*	—	! ! !	III
Jurassic.	22. **Marsupialia.** *Prodidelphia.*	22. **Didelphia.** *Didelphys.* *Perameles.*	—	! !	II
Cretaceous.	23. **Mallotheria.** *Prochoriata.*	23. **Insectivora.** Erinaceida. (Ictopsida +.)	=	!	I
Older Eocene.	24. **Lemuravida.** Older lemurs. Dentition 3. 1. 4. 3.	24. **Pachylemures.** *(Hyopsodus +.)* *(Adapis +.)*	≡	! ?	II
Neo-Eocene.	25. **Lemurogona.** Later lemurs. Dent. 2. 1. 4. 3.	25. **Autolemures.** *Eulemur.* *Stenops.*	≡	!	I
Oligocene.	26. **Dysmopitheca.** Western apes. Dent. 2. 1. 3. 3.	26. **Platyrrhinæ.** *(Anthropops +.)* *(Homunculus +.)*	—	!	II
Older Miocene.	27. **Cynopitheca.** Dog-faced apes (tailed).	27. **Papiomorpha.** *Cynocephalus.*	—	!	III
Neo-Miocene.	28. **Anthropoides.** Man-like apes (tail-less).	28. **Hylobatida.** Hylobates. Satyrus.	—	! !	III
Pliocene.	29. **Pithecanthropi.** Ape-men (alali, speechless).	29. **Anthropitheca.** Chimpanzee. Gorilla.	=	! ! !	III
Pleistocene.	30. **Homines.** Men, with speech.	30. **Weddahs.** Australian negroes.	—	! ! !	III

FIG. 8. Continuation of Figure 7 (Haeckel, 1905*a*, table 27).



SYNOPSIS OF THE CHIEF SECTIONS OF OUR STEM-HISTORY

FIRST SECTION OF OUR PHYLOGENY.

Man's Invertebrate Ancestors.

First phylogenetic stage: **The Protists.**

Man's ancestors are unicellular protozoa, originally unnucleated monera like the chromacea, structureless green particles of plasm; afterwards real nucleated cells (first plasmodomous *protophyta*, like the palmella; then plasmophagous *protozoa*, like the amœbæ).

Second phylogenetic stage: **The Blastæads.**

Man's ancestors are round cœnobia or colonies of protozoa; they consist of a close association of many homogeneous cells, and thus are individuals of the second order. They resemble the round cell-communities of the magosphæræ and volvocina, equivalent to the ontogenetic blastula: hollow globules. the wall of which consists of a single layer of ciliated cells (blastoderm).

Third phylogenetic stage: **The Gastræads.**

Man's ancestors are gastræads, like the simplest of the actual metazoa (prophysema, olynthus, hydra, pemmatodiscus). Their body consists merely of a primitive gut, the wall of which is made up of the two primary germinal layers.

Fourth phylogenetic stage: **The Platodes.**

Man's ancestors have substantially the organisation of simple platodes (at first like the cryptocœlic platodaria, later like the rhabdocœlic turbellaria). The leaf-shaped bilateral-symmetrical body has only one gut-opening, and developes the first trace of a nervous centre from the ectoderm in the middle line of the back (Figs. 293, 294).

Fifth phylogenetic stage: **The Vermalia.**

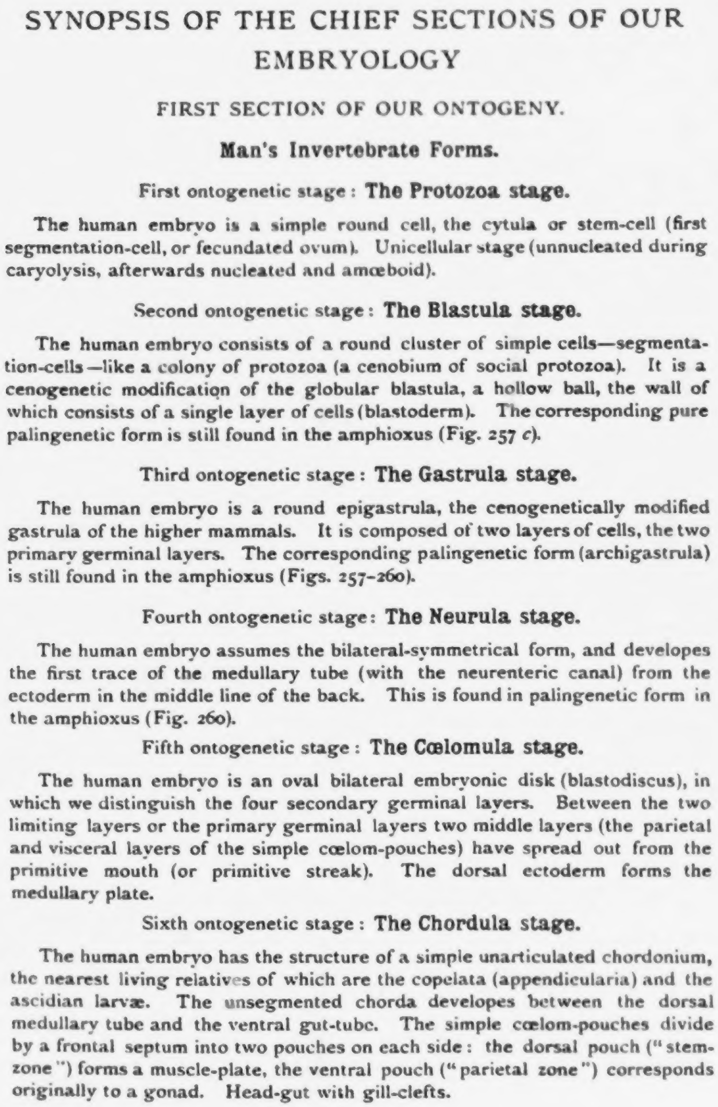
Man's ancestors have substantially the organisation of unarticulated vermalia, at first gastrotricha (ichthydina), afterwards frontonia (nemertina, enteropneusta). Four secondary germinal layers develop, two middle layers arising between the limiting layers (cœloma). The dorsal ectoderm forms the vertical plate, acroganglion (Fig. 297).

Sixth phylogenetic stage: **The Prochordonia.**

Man's ancestors have substantially the organisation of a simple unarticulated chordonium (copelata and ascidian larvæ). The unsegmented chorda developes between the dorsal medullary tube and the ventral gut-tube. The simple cœlom-pouches divide by a frontal septum into two on each side: the dorsal pouch (episomite) forms a muscle-plate; the ventral pouch (hyposomite) forms a gonad. Head-gut with gill-clefts.

FIG. 9. Figures 9 through 12 form a series comparing phylogeny and ontogeny in an abbreviated series. Figures 9 and 10 compare pre-vertebrate phylogeny and ontogeny (Haeckel, 1905*a*, table 36).

in Fig. 6, Stage 11 in Fig. 7, and Stage 6 in Fig. 9). Even here there was little useful information from paleontology (Fig. 7) and the hypothetical phylogeny had to be based mainly on morphological data, with an assist from embryology. The fossil data became more useful for later hypothetical stages (Fig. 8) and it is interesting to compare Haeckel's analysis with present-day information. In Figure 6 the sequence to mammals passes through the ancestors of these still-living forms: amphioxus, lamprey, shark, bony fish, and amphibian. It was only later that he realized that the last part of



SYNOPSIS OF THE CHIEF SECTIONS OF OUR EMBRYOLOGY

FIRST SECTION OF OUR ONTOGENY.

Man's Invertebrate Forms.

First ontogenetic stage: **The Protozoa stage.**

The human embryo is a simple round cell, the cytula or stem-cell (first segmentation-cell, or fecundated ovum). Unicellular stage (unnucleated during caryolysis, afterwards nucleated and amœboid).

Second ontogenetic stage: **The Blastula stage.**

The human embryo consists of a round cluster of simple cells—segmentation-cells—like a colony of protozoa (a cenobium of social protozoa). It is a cenogenetic modification of the globular blastula, a hollow ball, the wall of which consists of a single layer of cells (blastoderm). The corresponding pure palingenetic form is still found in the amphioxus (Fig. 257 *c*).

Third ontogenetic stage: **The Gastrula stage.**

The human embryo is a round epigastrula, the cenogenetically modified gastrula of the higher mammals. It is composed of two layers of cells, the two primary germinal layers. The corresponding palingenetic form (archigastrula) is still found in the amphioxus (Figs. 257–260).

Fourth ontogenetic stage: **The Neurula stage.**

The human embryo assumes the bilateral-symmetrical form, and developes the first trace of the medullary tube (with the neurenteric canal) from the ectoderm in the middle line of the back. This is found in palingenetic form in the amphioxus (Fig. 260).

Fifth ontogenetic stage: **The Cœlomula stage.**

The human embryo is an oval bilateral embryonic disk (blastodiscus), in which we distinguish the four secondary germinal layers. Between the two limiting layers or the primary germinal layers two middle layers (the parietal and visceral layers of the simple cœlom-pouches) have spread out from the primitive mouth (or primitive streak). The dorsal ectoderm forms the medullary plate.

Sixth ontogenetic stage: **The Chordula stage.**

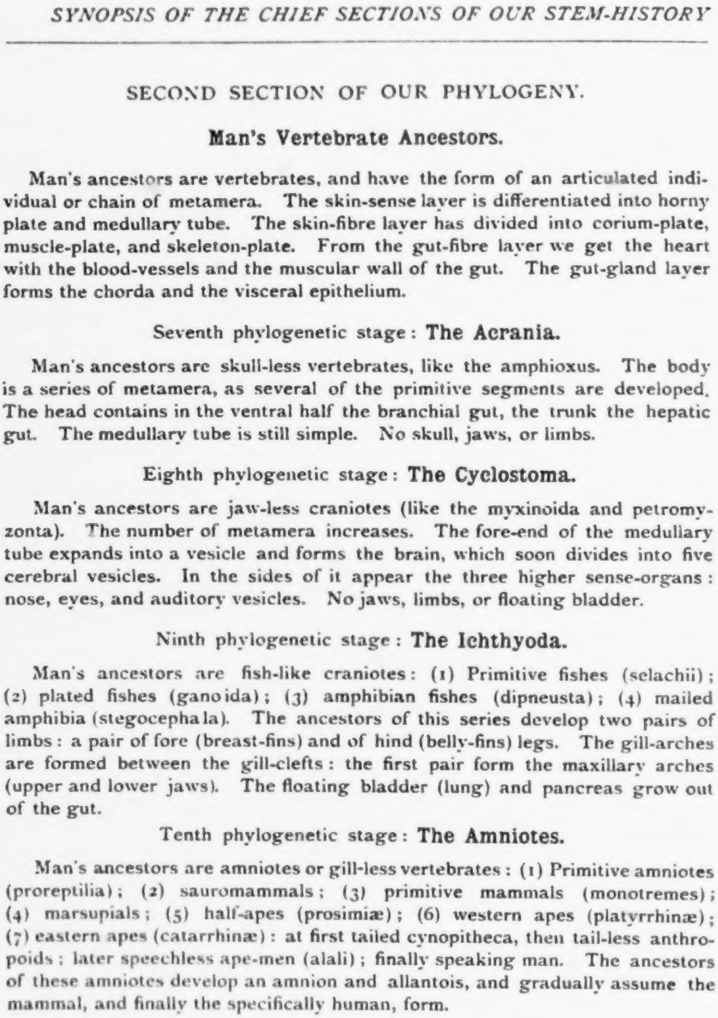
The human embryo has the structure of a simple unarticulated chordonium, the nearest living relatives of which are the copelata (appendicularia) and the ascidian larvæ. The unsegmented chorda developes between the dorsal medullary tube and the ventral gut-tube. The simple cœlom-pouches divide by a frontal septum into two pouches on each side: the dorsal pouch ("stem-zone") forms a muscle-plate, the ventral pouch ("parietal zone") corresponds originally to a gonad. Head-gut with gill-clefts.

FIG. 10. Compare with Figure 9 (Haeckel, 1905*a*, table 37).

the sequence should be amphibian—reptile—mammal (Fig. 8). Haeckel's sequence has stood the test of later discoveries well, at least so far as the vertebrates are concerned. The major difference is that the Selachii (Chondrichthyes) are thought to be derived from a very primitive group of bony fishes and are not themselves the most primitive fishes.

SACCULINA, BARNACLES, ASCIDIANS

More and more data seemed to suggest that early embryos may retain some relics of the basic structure of the group to which



SYNOPSIS OF THE CHIEF SECTIONS OF OUR STEM-HISTORY

SECOND SECTION OF OUR PHYLOGENY.

Man's Vertebrate Ancestors.

Man's ancestors are vertebrates, and have the form of an articulated individual or chain of metamera. The skin-sense layer is differentiated into horny plate and medullary tube. The skin-fibre layer has divided into corium-plate, muscle-plate, and skeleton-plate. From the gut-fibre layer we get the heart with the blood-vessels and the muscular wall of the gut. The gut-gland layer forms the chorda and the visceral epithelium.

Seventh phylogenetic stage: **The Acrania.**

Man's ancestors are skull-less vertebrates, like the amphioxus. The body is a series of metamera, as several of the primitive segments are developed. The head contains in the ventral half the branchial gut, the trunk the hepatic gut. The medullary tube is still simple. No skull, jaws, or limbs.

Eighth phylogenetic stage: **The Cyclostoma.**

Man's ancestors are jaw-less craniotes (like the myxinoida and petromyzonta). The number of metamera increases. The fore-end of the medullary tube expands into a vesicle and forms the brain, which soon divides into five cerebral vesicles. In the sides of it appear the three higher sense-organs: nose, eyes, and auditory vesicles. No jaws, limbs, or floating bladder.

Ninth phylogenetic stage: **The Ichthyoda.**

Man's ancestors are fish-like craniotes: (1) Primitive fishes (selachii); (2) plated fishes (ganoida); (3) amphibian fishes (dipneusta); (4) mailed amphibia (stegocephala). The ancestors of this series develop two pairs of limbs: a pair of fore (breast-fins) and of hind (belly-fins) legs. The gill-arches are formed between the gill-clefts: the first pair form the maxillary arches (upper and lower jaws). The floating bladder (lung) and pancreas grow out of the gut.

Tenth phylogenetic stage: **The Amniotes.**

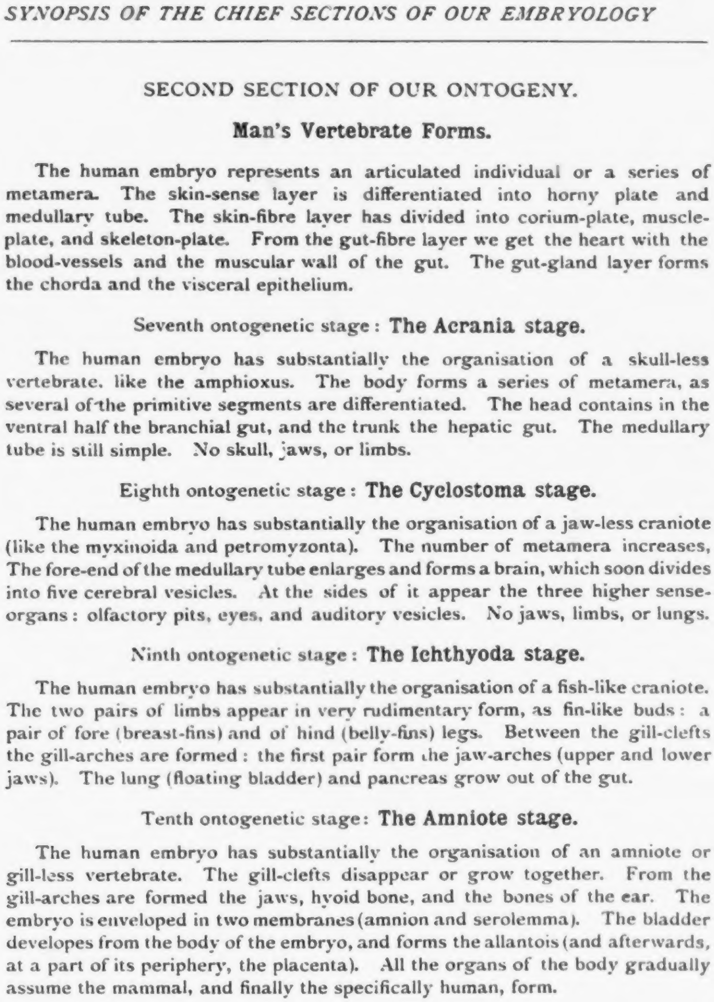
Man's ancestors are amniotes or gill-less vertebrates: (1) Primitive amniotes (proreptilia); (2) sauromammals; (3) primitive mammals (monotremes); (4) marsupials; (5) half-apes (prosimiæ); (6) western apes (platyrrhinæ); (7) eastern apes (catarrhinæ): at first tailed cynopitheca, then tail-less anthropoids; later speechless ape-men (alali); finally speaking man. The ancestors of these amniotes develop an amnion and allantois, and gradually assume the mammal, and finally the specifically human, form.

FIG. 11. The vertebrate stages in human phylogeny. Continuation of Figure 9 (Haeckel, 1905*a*, table 36).

they belong. If so, one might predict that a study of the embryos of species that were "problems" so far as their relationships were concerned would be productive.

Sacculina was the name given to a bag-like structure that could be found attached to various species of crabs. So far as external appearances are concerned it could be a tumor, especially since branching roots of the sac actually penetrate the host's abdomen. Closer study showed that the sac contains reproductive organs and some muscle and nerve tissue. Thus *Sacculina* could be considered a parasite and the branching roots that enter the host could be the mechanism for obtaining food.

One can not deduce from the structure of *Sacculina* what its affinities might be. A study of the early embryos, however, gave the answer. The eggs were found to develop into a well known larval type—the nauplius larva with three pairs of appendages—that



SYNOPSIS OF THE CHIEF SECTIONS OF OUR EMBRYOLOGY

SECOND SECTION OF OUR ONTOGENY.

Man's Vertebrate Forms.

The human embryo represents an articulated individual or a series of metamera. The skin-sense layer is differentiated into horny plate and medullary tube. The skin-fibre layer has divided into corium-plate, muscle-plate, and skeleton-plate. From the gut-fibre layer we get the heart with the blood-vessels and the muscular wall of the gut. The gut-gland layer forms the chorda and the visceral epithelium.

Seventh ontogenetic stage: **The Acrania stage.**

The human embryo has substantially the organisation of a skull-less vertebrate, like the amphioxus. The body forms a series of metamera, as several of the primitive segments are differentiated. The head contains in the ventral half the branchial gut, and the trunk the hepatic gut. The medullary tube is still simple. No skull, jaws, or limbs.

Eighth ontogenetic stage: **The Cyclostoma stage.**

The human embryo has substantially the organisation of a jaw-less craniote (like the myxinoida and petromyzonta). The number of metamera increases, The fore-end of the medullary tube enlarges and forms a brain, which soon divides into five cerebral vesicles. At the sides of it appear the three higher sense-organs: olfactory pits, eyes, and auditory vesicles. No jaws, limbs, or lungs.

Ninth ontogenetic stage: **The Ichthyoda stage.**

The human embryo has substantially the organisation of a fish-like craniote. The two pairs of limbs appear in very rudimentary form, as fin-like buds: a pair of fore (breast-fins) and of hind (belly-fins) legs. Between the gill-clefts the gill-arches are formed: the first pair form the jaw-arches (upper and lower jaws). The lung (floating bladder) and pancreas grow out of the gut.

Tenth ontogenetic stage: **The Amniote stage.**

The human embryo has substantially the organisation of an amniote or gill-less vertebrate. The gill-clefts disappear or grow together. From the gill-arches are formed the jaws, hyoid bone, and the bones of the ear. The embryo is enveloped in two membranes (amnion and serolemma). The bladder developes from the body of the embryo, and forms the allantois (and afterwards, at a part of its periphery, the placenta). All the organs of the body gradually assume the mammal, and finally the specifically human, form.

FIG. 12. Compare with Figure 11 (Haeckel, 1905*a*, table 37).

is characteristic of many crustaceans. Later the nauplius larva transforms into the cypris larva, again a familiar crustacean larval type. After a period of independent life the cypris larva attaches to a crab, loses its appendages and most of its anatomy for that matter, and becomes the tumor-like structure of the adult *Sacculina*. Figure 15 shows a variety of nauplius larvae of crustaceans. As you can see, that of *Sacculina* fits the picture.

As noted before the story is similar for the barnacles, a large group of invertebrates. Since they are covered by a shell, many early naturalists considered them to be mollusks. A study of the embryos, however, showed that the barnacles are crustaceans, with a typical crustacean larval type (Fig. 15, D—*Lepas*).

The answer for the ascidians was different but the method of obtaining the answer was the same. The ascidians, or tunicates, are marine organisms. Most of the common ones look like an amorphous mass of

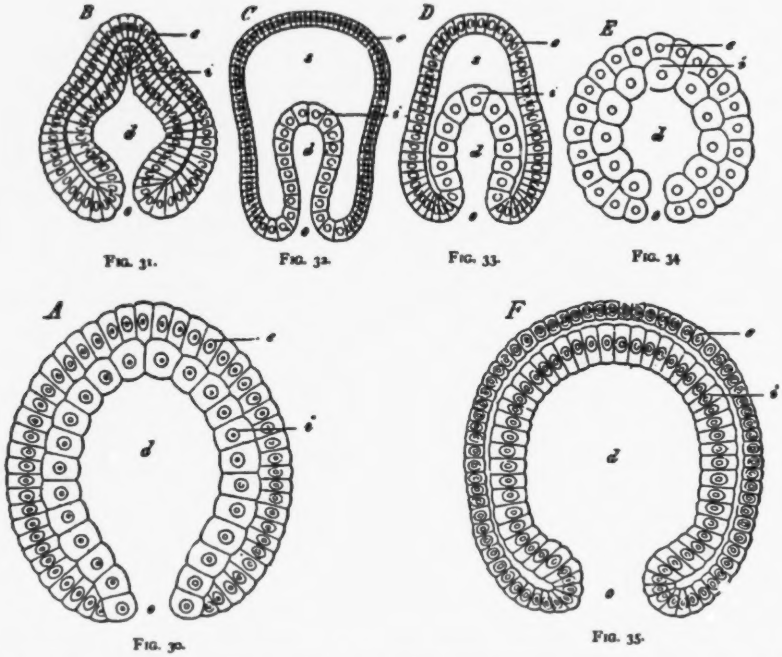


FIG. 13. The Gastraead level of organization. A is a hypothetical simple two-layered animal, or Gastraead. It is similar to the gastrula stage of many metazoans having eggs with little yolk: the arrow-worm, *Sagitta* (B); the starfish, *Uraster* (C); the crab, *Nauplius* (D); the pond snail, *Limnaeus* (E); and the lancet, *Amphioxus* (F). In each figure *d* is the archenteron, *o* the mouth, *s* the blastocoel, *i* the endoderm, and *e* the ectoderm (Haeckel, 1905*b*, p. 63).

"something" that is attached to wharf pilings, rocks, etc. The adults consist mostly of a basket with perforated walls. Water enters an opening and passes through the walls of the basket and food particles are strained out. Again it would be difficult to classify these objects on the basis of the structure of the adult. The larvae provide the answer—they are chordates, with a nerve tube, pharyngeal gill slits, and a notochord.

Clearly ontogeny was a powerful tool for discovering relationships among organisms.

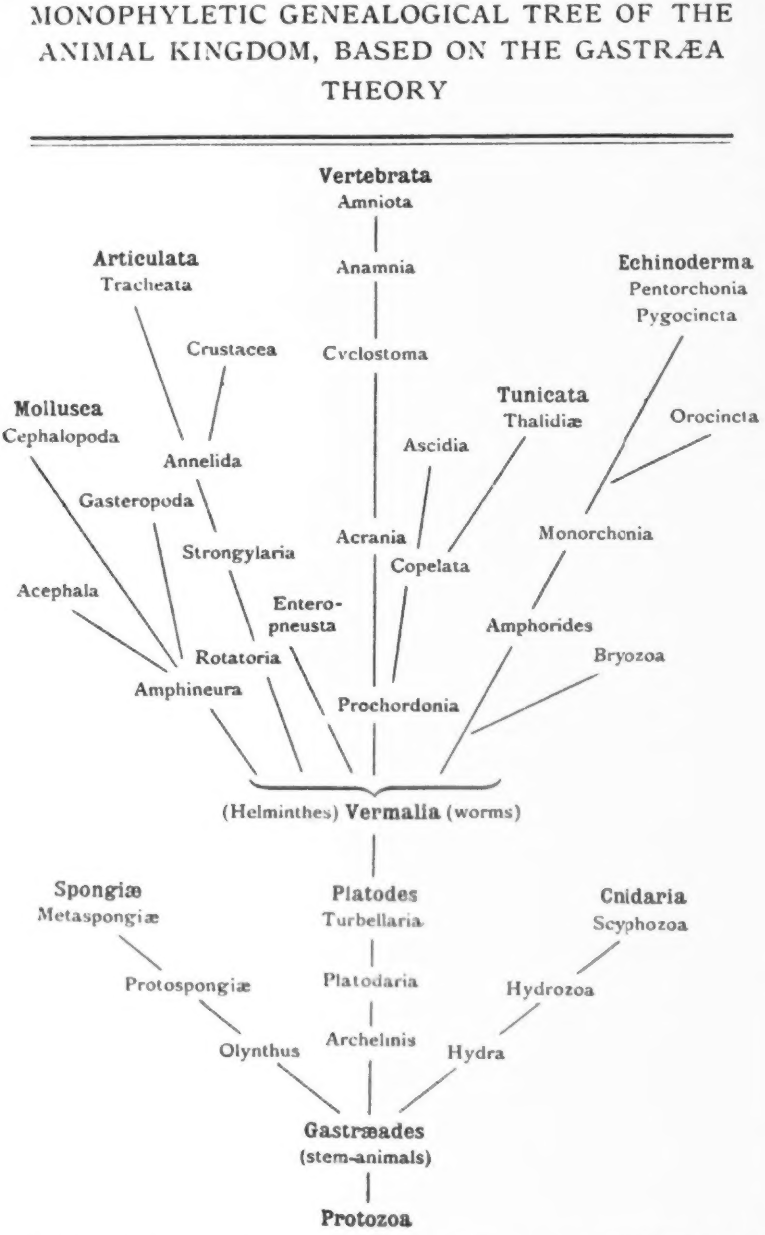
RECAPITULATION EVALUATED

Figures 6–12 and 13 represent a bold attempt by Haeckel to reduce to a single concept a very large amount of data—an attempt made when the data were most inadequate. He speculated far beyond the data available to him but in so doing he provided hypotheses for others to test and suggested studies that were worth doing. But this had serious disadvantages and in Oppenheimer's (1955, p. 15) opinion,

> What was damaging to science was Haeckel's fervency to oversimplify all morphology through his biogenetic law that "die Ontogenie ist eine Recapitulation der Phylogenie."

Nevertheless it is surprising how many of his ideas turned out to be correct in general—though rarely in detail. For example, his notions about the origin of life are not too different from the hypotheses of today. His attempt to interrelate all kinds of animals (Fig. 14) would not be accepted by all biologists today but, for that matter, no other scheme has achieved widespread approbation.

There are parallels between Darwin and Haeckel in the general acceptance of their



MONOPHYLETIC GENEALOGICAL TREE OF THE ANIMAL KINGDOM, BASED ON THE GASTRÆA THEORY

Vertebrata
Amniota
Anamnia
Cyclostoma
Acrania
Articulata
Tracheata
Crustacea
Annelida
Strongylaria
Echinoderma
Pentorchonia
Pygocincta
Orocincta
Monorchonia
Amphorides
Tunicata
Thalidiæ
Ascidia
Copelata
Mollusca
Cephalopoda
Gasteropoda
Acephala
Amphineura
Rotatoria
Entero-pneusta
Prochordonia
Bryozoa
(Helminthes) Vermalia (worms)
Spongiæ
Metaspongiæ
Protospongiæ
Olynthus
Platodes
Turbellaria
Platodaria
Archelminis
Cnidaria
Scyphozoa
Hydrozoa
Hydra
Gastræades
(stem-animals)
Protozoa

FIG. 14 Haeckel's hypothesis for the interrelations of the major groups of animals (Haeckel, 1905*a*, table 25).

main theses and the rejection of many of the details. Most biologists agreed that Darwin had shown beyond all reasonable doubt that evolution had occurred but his suggested mechanisms—spontaneous variations acted upon by natural selection—were thought improbable or impossible until well into the 20th century. Haeckel's

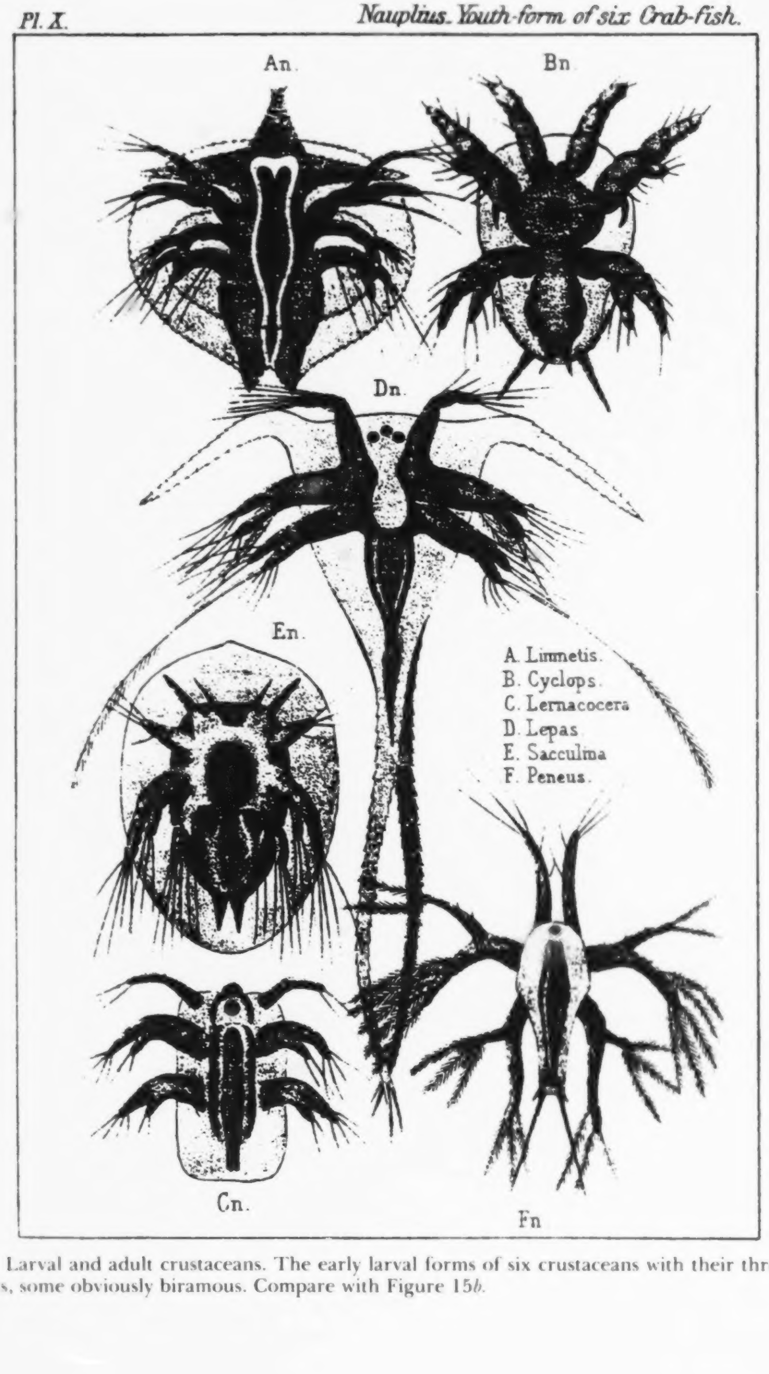

Pl. X.
Nauplius. Youth-form of six Crab-fish.
An.
Bn.
Dn.
En.
A. Limnetis.
B. Cyclops.
C. Lernacocera
D. Lepas.
E. Sacculina
F. Peneus.
Cn.
Fn

FIG. 15*a*. Larval and adult crustaceans. The early larval forms of six crustaceans with their three pairs of appendages, some obviously biramous. Compare with Figure 15*b*.

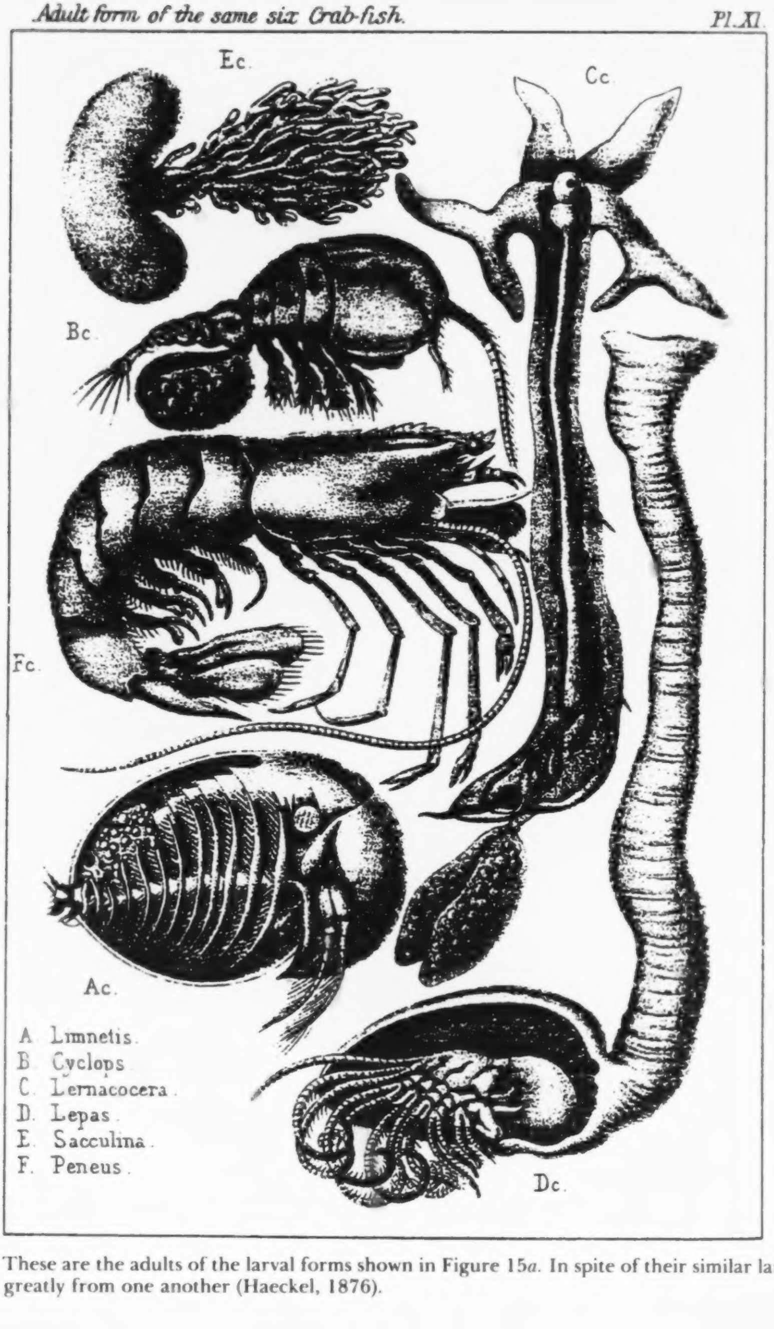

Adult form of the same six Crab-fish.
Pl.XI.
Ec.
Cc.
Bc.
Fc.
Ac.
Dc.
A. Limnetis.
B. Cyclops.
C. Lernacocera.
D. Lepas.
E. Sacculina.
F. Peneus.

FIG. 15*b*. These are the adults of the larval forms shown in Figure 15*a*. In spite of their similar larval forms they differ greatly from one another (Haeckel, 1876).

synthesis of the data of descriptive embryology, evolution, and comparative anatomy is accepted in theory even though there has been a strong reaction against the details. Many have claimed that Haeckel believed that embryos recapitulate the *adult* stages of ancestors. I read Haeckel differently, possibly being biased by admiration for his attempts at synthesis. Surely one cannot gather from his discussion of the "Ichthyoda" stage in our phylogeny (Fig. 11) and in ontogeny (Fig. 12) that Haeckel is suggesting that we recapitulate that stage by swimming around in the amnion with pectoral and pelvic fins, a fishy tail, and encased in a scaly skin.

Gould (1977) has provided a magnificent synthesis of ideas related to recapitulation. He writes

> The theory of recapitulation played a fundamental role in a host of diverse disciplines; I suspect that its influence as an import from evolutionary theory into other fields was exceeded only by natural selection itself during the nineteenth century . . . . We grasp the importance of recapitulation only when we understand that it served as the organizing idea for generations of work in comparative anatomy, physiology, and morphology . . . . In my own field of paleontology, for example, it governed most studies in phyletic reconstruction from Haeckel's day right through the 1930s (pp. 115–116).

E. S. Russell (1917, pp. 312–313) offers another useful perspective:

> But evolutionary morphology for all practical purposes was a development of pure or idealistic morphology, and was powerless to bring to fruit the new conception with which evolution-theory had enriched it. The reason is not far to seek. Pure morphology is essentially a science of comparison which seeks to disentangle the unity hidden beneath the diversity of organic form. It is not immediately concerned with the causes of organic diversity—that is rather the task of the sciences of the individual, heredity and development. To take an example—the recapitulation theory may legitimately be used as a law of pure morphology, as stating the abstract relation of ontogeny to phylogeny, and the probable line of descent of any organism may be deduced from it, as a mere matter of the ideal derivation of one form from another; but an explanation of the reason for the recapitulation of ancestral history during development can clearly not be given by a pure morphology unaided. From the fact that the common starfish shows in the course of its development distinct traces of a stalk it is possible to infer, taking other evidence also into consideration, that the ancestors of the starfish were at one stage of their existence stalked and sessile organisms [perhaps resembling crinoids]. But this leaves unanswered the question as to how and why the starfish does still repeat after so many millions of years part of the organisation of one of its remote ancestors. Why is this feature retained, and by what means has it been conserved through countless generations? It is clear that the answer can be given only by a science of the causes of the production and retention of form, by a causal morphology, based upon a study of heredity and development.
>
> From the point of view of the pure morphologist the recapitulation theory is an instrument of research enabling him to reconstruct probable lines of descent; from the standpoint of the student of development and heredity the fact of recapitulation is a difficult problem whose solution would perhaps give the key to a true understanding of the real nature of heredity.
>
> To make full use of the conception of the organism as an historical being it is necessary then to understand the causal nexus between ontogeny and phylogeny.

Summary. But as we see it today, von Baer came closer to an acceptable concept than Haeckel did. Taking the best known examples, the chordates, we must admit that embryos do not in general recapitulate the adult stages of their ancestors. Chordate embryos do share a common plan of development that has an early stage with notochord, dorsal nerve tube, and pharyngeal gill slits or pouches separated by gill arches (I, pp. 502–503). The adults of the lower chordate classes change less from this fundamental plan than the adults of the higher classes. The higher forms *retain* some fundamental features in their early development and then differentiate in their special ways. Thus the organ systems of the amphibians, reptiles, birds, and mammals can be understood as variations on a pattern based on the morphology of agnathans and primitive fish. Good examples are the pronephros–mesonephros–metanephros series of kidneys (I, pp. 503–504) and the jaw bones and ear ossicles (I, pp. 408–409).

It must be kept in mind that it is essentially impossible to show beyond all reasonable doubt that ontogeny does recapitulate phylogeny for structures that do not fossilize. Comparisons of living organisms cannot substitute for true ancestors. Thus tests of the hypothesis of recapitulation must be based on skeletons or other structures that form the fossil record. Mammalian ear ossicles are a case in point. Mainly on the basis of embryological observations, C. B. Reichert, in 1837, suggested their homologies to the jaw bones of lower forms (I, pp. 498–499). A century later this bold hypothesis was to be fully confirmed by the discovery of a series of fossils of the mammal-like reptiles.

The very difficult problem of why such "useless" structures as the notochord, gill pouches, or pronephros are recapitulated would not be answered until the organizer theory was developed.

Haeckel's hypothetical prechordate ancestors did not find universal acceptance. Nevertheless his effort was eminently worthwhile. The problem is important and to this day there are no satisfactory answers or known techniques for obtaining them. At least Haeckel suggested how one *might* think about prechordate ancestors and we may have to settle for that. It is certainly reasonable to think that early metazoans may have passed first through a stage similar in general structure to a planula larva and then later through a stage similar to a gastrula. The chance of discovering a planula-like or gastrula-like ancestor in rocks laid down two billion years ago seems remote and, if such were found, to make a convincing argument that it was ancestral to the metazoan phyla would be almost impossible. But stranger and luckier things than that have happened.

There seems to be little question that Haeckel extended the concept of recapitulation well beyond the point where it could be tested in his day or even ours. It was an imaginative attempt and elements were soon regarded as improbable or wrong. Nevertheless the concept remains a powerful and useful way of trying to understand the structure of organisms. Data such as shown in Haeckel's illustration reproduced as Figure 16 are difficult to understand without the concept of recapitulation. So also are the many examples of vestigial structures. It is far simpler to account for the human appendix as a relic of an essential structure of an ancestor rather than as an object evolved mainly to assist the cash-flow problems of our surgeons.

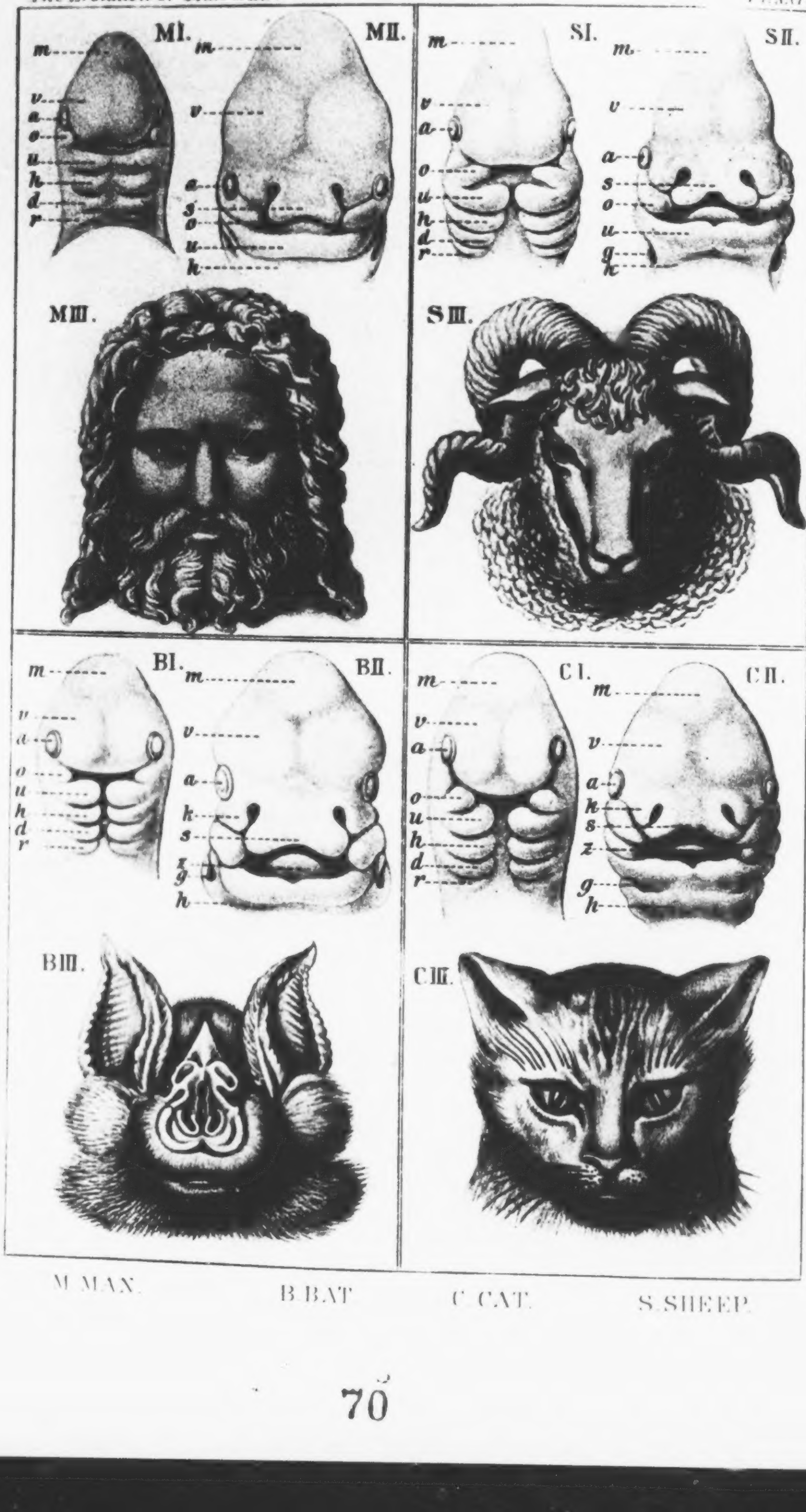
So to paraphrase an earlier quotation of Stephen Gould's, "Let's not throw recapitulation out with Haeckel." In fact, we should keep both but be cautious in the manner in which they are used.

COMPARATIVE EMBRYOLOGY

In 1880–1881 Francis Balfour's two volume *A Treatise on Comparative Anatomy* was published. Apart from a briefer work on the same subject by Packard (1876), this was the first major synthesis since von Baer. In that half century enormous progress had been made in embryology. There were more students of development and better tools and methods: better microscopes, better histological techniques, and improved methods for obtaining and handling embryological material. As a result data became available on the developmental patterns of species belonging to all major groups of animals. Advances in Cell Theory allowed a deeper understanding of embryos—embryos were recognized as composed of cells, and the results of an

THE EMBRYONIC DEVELOPMENT OF THE FACE.

The Evolution of Man V Ed. Pl. XXIV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M. MAN. B. BAT. C. CAT. S. SHEEP.

orderly process of cell division. The Theory of Evolution provided an explanatory hypothesis for the similarities as well as the variations in patterns of development. The data of embryology made so much sense in terms of evolution that they became one of the better evidences for evolution itself. And, in the absence of data from extinct ancestors, embryos were searched for evidences of phylogeny.

Quite apart from these research goals, there was the hope that uniformities, that is, rules that would hold for many different sorts of development, could be found. Had one known nothing of the varieties of ontogenies to be discovered, it might have been predicted that patterns of development would be as varied as patterns of adult morphology. Such proved not to be the case. The remarkable similarities in development of vertebrates, known to observers from Aristotle to Haeckel, was found to hold for invertebrates as well. There were, indeed, rules and the most diverse ends were achieved by variations on a fundamental pattern. It was discovered that, throughout the metazoans, one could usually recognize these major steps in development.

1. Development begins with the activation of an ovum by a sperm (sexual reproduction) or by other means (parthenogenesis).

2. The activated ovum divides repeatedly, and comparatively rapidly, about 8 to 12 times by mitosis. The result is a ball of cells, the blastula, with a cavity, the blastocoel.

3. There then ensues a rearrangement of cells with some moving inward forming a cavity, the archenteron. The cavity has an opening to the outside, the blastopore. This cup-shaped structure with an inner and outer layer is the gastrula (Fig. 13), which was regarded by Haeckel as the basic body plan from which all metazoans evolved (Fig. 14). The rate of cell division slows by the gastrula stage and remains slow for the rest of development.

4. The rearrangement of cells results in the formation of the embryonic layers. There are two of these in the coelenterates, an outer ectoderm and an inner endoderm. Other metazoans have an additional layer, the mesoderm, between the ectoderm and endoderm. When these layers first become definite their cells are essentially the same.

5. As development continues there is an increase in cell number, the cells become visibly differentiated, and there are structural rearrangements of the cells leading to the formation of organs and tissue layers.

6. Throughout the metazoans there is considerable uniformity in the structures developing from each germ layer. Typically the skin, nervous system, and some types of excretory organs are derived from the ectoderm; the lining of the alimentary canal and the associated organs are derived from endoderm; the circulatory system, muscles, connective tissue, and some types of excretory organs are derived from the mesoderm.

This commonality of patterns of development found its formal explanation in the theory of evolution and its derivative, recapitulation.

Nevertheless it was discovered that there are two strikingly different patterns of development—direct and indirect—that result in similar end products. One of the main reasons appeared to be the quantity of yolk in the ovum or the availability of food directly from the mother. The ova of some species, human beings and sea urchins for example, contain very small amounts of yolk—far less than is required to carry the embryo to the juvenile stage. Such embryos must rely on external sources of food—either capturing it themselves or obtaining it from mother.

The sea urchin mode of development,

←

FIG. 16. Early embryos and adults of four mammals. The embryos were much the same in spite of their very different destinies as von Baer and other embryologists had discovered (Haeckel, 1905*a*, p. 290).

which is common among the invertebrates, is for the embryo rapidly to reach a free-living larval stage, in this case a pluteus. The pluteus is a microscopic larva that obtains its food from the ocean. It bears no resemblance to the adult. It swims, feeds and grows. Eventually a complete restructuring of its anatomy, physiology, and life style begins—it undergoes metamorphosis into the adult sea urchin. This is called *indirect development,* since the embryo does not develop directly into an adult but passes through a larval stage very different in structure, physiology, and behavior from the adult.

Human beings and other mammals rely on nourishment from the mother. They differentiate into the juvenile form without a free-living, food-capturing larval stage. These embryos have *direct development.*

Direct development also occurs in birds but there the source of the food supply is different—the ovum contains sufficient food to carry the embryo to the juvenile stage.

The quantity of yolk not only determines the pattern of development in oviparous species but some of the details of early development as well. In species with little yolk, mitosis divides the entire embryo into cells of roughly equal size and a "typical" gastrula (Fig. 13) is formed. In many familiar frog species there is an intermediate quantity of yolk and the pattern of cleavage and gastrulation is much modified. In species with large amounts of yolk, such as the birds, the pattern of cleavage, gastrulation, and organ formation is so different from that of species with less yolk that much study was required before the basic homologies could be understood.

Patterns of direct and indirect development are obviously exceedingly different. It came as a distinct surprise to embryologists, therefore, to find that both patterns could occur in closely related species. Try to imagine how a strict recapitulationist would have interpretated these observations.

Most species of anurans of the United States and Europe—the ones that were studied first—have eggs with a modest amount of yolk and indirect development. The early embryo develops into a free-living tadpole, with gills and a tail, that seeks food in its aquatic environment. After a period varying from weeks to years, depending on the species, there ensues a drastic metamorphosis during which the tail is resorbed, limbs appear, the entire anatomy undergoes considerable modification, and a froglet hops out on land. These frog species, then, seem to recapitulate their fishy ancestors in their tadpole stage.

As more and more frog species were studied, however, it became clear that some have huge eggs and direct development, that is, the tadpole stage is entirely omitted and the embryo develops straight into a froglet (Duellman and Trueb, 1986, ch. 2; Salthe and Mecham, 1974). In these cases the anuran embryo is similar to birds in having enough yolk to last to the juvenile stage. Duellman and Trueb estimate that "direct development must have evolved independently in at least 12 groups" of anurans.

It must be emphasized that the adults of those species of frogs that develop directly and indirectly—seemingly fundamentally different patterns—may belong to the same genus and, hence, be very much alike. This same phenomenon exists in salamanders as well. *Desmognathus fuscus* has indirect development with a feeding larval stage whereas the closely similar *Desmognathus wrighti* has direct development.

It might be rewarding to explore with students where these data leave the concept of recapitulation. Should we conclude that *Desmognathus fuscus* and *Desmognathus wrighti* had basically different evolutionary histories?

This is a case where one must consider how anomalous data relate to the main body of data. When this is done there seems to be little reason to change one's opinion about the usefulness of the concept of recapitulation. The embryos of both species exhibit in their early stages the structures that make the concept of recapitulation a useful way to account for the data. They have the basic chordate structures: notochord, dorsal nerve tube, and pharyngeal pouches. Their circulatory systems, jaw bones, and kidneys show the same modifications of the basic vertebrate body plan. The data are evaluated, therefore, and one bases relationships on those thought to be most important.

Thus, the patterns of direct and indirect development may not be so fundamentally different as we first thought. This conclusion is strengthened by the probability that direct development may have arisen independently those 12 times.

It bears repeating that the concept of recapitulation offers an explanation for only some aspects of development. These are the situations where vestiges of ancient patterns of development appear to have persisted. It must be accepted that these are generally restricted to the earliest stages of development. The concept is of value because it can reasonably account for some otherwise inexplicable observations.

We still have the problem, however, of discovering why any ancestral structures persist.

THOSE GERM LAYERS

The traditional treatment of embryology in beginning courses stresses the details of development. If the students are left with any organizing concept at all it is that the outside of the embryo is blue, the middle section red, and the inside yellow. Seemingly that concept has universal applicability for the organisms considered and, by inference, for those that were not.

And the Theory of Germ Layers has been one of the mainstays of descriptive embryologists as well as of students. The concept grew slowly from Wolff, Pander, and von Baer to its extensive development by Haeckel (1905*b*, ch. 10) and Lankester (1877). It has been almost as contentious as the concept of recapitulation. The arguments have been mainly about the applicability of the concept to embryos of different phyla and what is implied about the developmental potential of the layers themselves.

Let us consider those two aspects separately. If one is saying only that metazoan embryos consist of two (coelenterates) or three (the other major phyla) layers during an early embryonic stage, the concept has great heuristic value: "among the higher Metazoa there is then a wide correspondence between the germ layers as regards their fate and function in ontogeny" (Hyman, 1940, p. 270). It is conceptually satisfying to know that in animals differing greatly from one another the skin, the most anterior and most posterior parts of the alimentary canal, and the nervous system develop from the ectoderm; the muscles, connective tissues, skeletal and circulatory systems (if there are any), from the mesoderm; and, except for the two ends, the lining of the digestive system and its associated glands from the endoderm.

While "wide correspondence" exists between what the germlayers do, that does not signify that they are homologous. Nevertheless the origins of the three layers are so much alike throughout the vertebrates that it can be said that they are homologous by virtue of identity of embryonic origin. The conclusive data, origin from the same part of an ancestral species, will most likely never be available.

One can go one step further and entertain the hypothesis that there is a basic homology of germ layers throughout the Metazoa. In 1849 Thomas Henry Huxley started this line of inquiry and later wrote (1878, pp. 110–114) that the fundamental structure of a coelenterate consists essentially of

> a sac having at one end an ingestive or oral opening, which leads into a digestive cavity. The wall of the sac is composed of two cellular membranes, the outer of which is termed the *ectoderm*, and the inner the *endoderm*, the former having the morphological value of the epidermis of the higher animals, and the latter that of the epithelium of the alimentary canal . . . . The peculiarity in the structure of the body walls of the *Hydrozoa* [a Class of Coelenterata], to which I have just referred, possesses a singular interest in its bearing upon the truth (for, with due limitation, it is a great truth) that there is a certain similarity between the adult states of lower animals and the embryonic conditions of those of higher

> organization . . . . Thus there is a very real and genuine analogy between the adult Hydrozoön and the embryonic vertebrate animal; but I need hardly say it by no means justifies the assumption that the Hydrozoa are in any sense "arrested developments" of higher organisms. All that can justly be affirmed is, that the Hydrozoön travels for a certain distance along the same great highway of development as the higher animal, before it turns off to follow a road which leads to its special destination.

The mid 19th century argument, especially after Haeckel proposed his Gastraea Theory, for the homology of these germ layers was something like this. The basic structure of a hydrozoan is that of a double-walled vase, the central cavity being the enteron. The gastrula stage of an idealized vertebrate is essentially the same. No vertebrate embryo exhibits such an idealized structure but that difficulty can be explained, in part, as being due to yolk modifying development. The gastrula of amphioxus (Fig. 13F) does come close to the idealized two-layered vase. We may suspect, therefore, that the similarity of the two-layered hydrozoan body and the two-layered archetypal vertebrate gastrula makes tenable the homology of their germ layers.

That argument is far less compelling in the late 20th century. As more information became available, it was clear that the germ layers arise in many different ways in the metazoans. Therefore one cannot use identity of embryonic origin as proof of homology since the origins are not identical. One cannot maintain, for example that the mesoderm is the "same thing" throughout the bilateral phyla. To what extent can the cells on the outside of an earthworm be considered homologous to the cells on the outside of a starfish? Any answer is as dubious as would be any clear notion of how one would find out.

Once the germ layers have been formed, however, there is great uniformity in what they do, as Hyman noted. Therein lies their conceptual and pedagological importance.

A second interesting problem has to do with the relation between what the germ layers form in the course of development and their innate abilities. Is there something "mesodermal" about the mesodermal cells, meaning that they produce only mesodermal organs, and that mesodermal organs are produced only by the mesodermal layer? Questions of this type can be formulated in hypotheses that can be tested. As we will learn later, the Spemann school found that there is no restriction on what mesodermal cells can form and mesodermal structures can be formed from other than mesodermal cells.

Other evidence of the non-specificity of the germ layers comes from studies of regeneration where in some cases the structures of the regenerated individual are derived from different germ layers than those from which they were first formed in embryonic development.

What does this tell us about the usefulness of the Theory of Germ Layers for students in first-year courses? Libbie Hyman, one of the greatest students of invertebrates of all time, answers that question as follows:

> It can scarcely be doubted that the later stages of development exhibit a certain similarity especially in the Bilateria [metazoans other than sponges, coelenterates, and ctenophores] and that in general each germ layer gives rise to certain definite organs. The doctrine of the homology of the germ layers may therefore be considered as broadly acceptable and if applied with caution may be used in interpreting embryological facts. It must always be borne in mind that a developing embryo is living, plastic, and modifiable, responding to changed conditions by morphological changes. Probably no development at present adheres to its original course, but all ontogenies have undergone changes, such as shortening of some stages, prolongation of others, precocious development of certain parts (heterochronism), and production of larval organs adapted to a free-swimming life. We may assume a general tendency toward cutting short and condensing stages no longer essential to the life of the embryo or to the development of future organs, and toward the pre-

> cocious or new appearance of useful parts. Every ontogeny is a compromise between an inherited ancestral mode of development and adaptive modifications and adjustments (1940, pp. 271–272).

In her fine study of germ-layer specificity Jane Oppenheimer (1940) makes it abundantly clear that the cells of germ layers do not have innate specificity. Apart from this however, the concept of germ layers is of some significance:

> It seems certain that the precise location of a cell during gastrulation in many forms, or the precise origin of its cytoplasm from the egg in others, is in many cases correlated with the type of its later activity; therefore in a certain sense the germ-layers are of topographic significance, since the cells pass through them in their orderly progression of movements. In a teleological sense, formation of germ-layers seems to be the embryo's method of sorting out its constituent parts. The essential point is, however, that this method is not the only method that the embryo can call upon to attain a specific end, and here as in many other cases in development the embryo can, when necessary, modify or abandon one method in favor of another . . . . The task of the student of the germ-layers then must become more than an attempt to discern how the embryo sorts its cells into one layer or another; it must become an elucidation of how wide the potencies of the germ-layers become subject to limitation to their normal accomplishments.

So, for the student the concept of germ layers should be considered no more than a map to guide the study of normal development; for the developmental biologist the germ layers should be the basis of experiments to throw light on the processes of differentiation.

The last half of the 19th century saw embryologists interpreting their observations on normal development in relation to the Theory of Evolution. Descriptive embryology, so interpreted, was the dominant paradigm. Frederick B. Churchill (1986, p. 7) has provided a fitting closing statement to our discussion of this era of embryology.

> When the historian of biology turns to nineteenth century embryology, he conjures forth an imposing structure. At one end of the century exist the exemplary observations of Pander and von Baer and at the other the dramatic experiments of Roux and Driesch. Firmly settled between these two opposing buttresses rises the towering edifice of classical descriptive embryology, solid in its discoveries, magnificent in its tracery and fine details, and as defiant of and removed from modern biology as a gothic cathedral is from today's secular world. Few can doubt the real achievements of those artisans who, in constructing this temple, eternally glorified the perseverance and perspicacity of descriptive biologists. From von Baer's discovery of the mammalian ovum, on through Rathke's analysis of the branchial arches, Müller's, Reichert's and Huxley's examination of the development of the vertebrate skeletal system and the exquisite descriptions of invertebrate development by Kowalevsky, Metchnikoff and Kleinenberg, and terminating with the monumental studies on the development of single organisms or organ systems by Götte, Balfour, Semper, His and scores of others, the spires of this cathedral rest on the surest of pillars.

Descriptive embryology of the 19th century was embryology looking outward—relating the phenomena of development to the basic biological concept of all time. Concurrently, however, there were tentative beginnings of another paradigm—analytical embryology. This is embryology looking inward—attempting to understand the causal relations in development. Analytical embryology is the paradigm that demands our attention today.

References: Descriptive embryology—von Baer to Haeckel

Adelmann (1966), Agassiz (1849, 1859), von Baer (1827, 1828), Balfour (1880–1881), Bather (1893), Baxter (1977), de

Beer (1958), Churchill (1970*b*, 1986), Di Gregorio (1984), Gardner (1965), Garstang (1922), Gasking (1967), George (1933), Haeckel (1866, 1868, 1874, *1876, *1905*a*), T. S. Hall (1951), O. Hertwig (1901), Horder *et al.* (1986), Huxley (1849, 1878), Hyman (1940), Kohlbrugge (1911), Kölliker (1861), Korschelt and Heider (1895), Lankester (1873, 1877), Lovejoy (1959), Magner (1979), Maienschein (1978), Mayr (1982), Meyer (1956), Oppenheimer (1940, 1955, 1957, 1959*a*, 1959*b*, 1963, 1964, 1966, *1967, 1970*a*, 1973), Ospovat (1976), Packard (1876), Reichert (1837), Rinard (1981), E. S. Russell (1917, 1930), Sarton (1931), Sedgwick (1894), Singer (1950), and E. B. Wilson (1896*a*, 1899).

THE AMPHIBIAN EMBRYO—EXTERNAL DEVELOPMENT

When the main reason for studying embryos began to switch from seeking to learn about evolution to an analysis of the causal factors in development, the amphibians were found to provide excellent material. Prior to that switch, it was important to have embryological data from a broad sample of organisms. For analytical embryology, on the other hand, it was necessary to use species with embryos that could survive experimental manipulations. The mature eggs of many common European and North American frogs and salamanders are usually about two or three millimeters in diameter, hence, large enough to be operated upon. Their embryos are hardy and heal well and recovery can usually be expected following operations and other experimental procedures. Each fertilized egg has a supply of yolk granules sufficient to carry the embryo to a free-living stage. This is a great advantage since the difficult problem of supplying an external source of food is avoided.

Much of the early data on the analysis of development came from amphibian embryos so it is necessary to give a short synopsis of the main events in their development. The following description is of embryos of *Rana pipiens* from Vermont. This species and other meadow frogs very similar to it are widely distributed in North and Central America. For several generations they have been extensively used in experiments. The external aspects of development, as shown in Figures 17–23, will be described first.

The rate of development depends on temperature—the embryos shown in the illustrations were kept at a constant temperature of 20°C. The numbers on each photograph give the time in hours after fertilization. Had the embryos been kept at 25°, development would have required about half the time, and if kept at 15° nearly twice as long. The lowest and highest temperatures for normal development are, respectively, about 5° and 28°. The embryos in the illustrations are magnified about 25 times.

Breeding. In the spring, spurred by warming days, moist nights, and hormonal changes, males and females congregate in ponds and swamps for a brief breeding period. At this time all of the mature ova of the females leave the ovary, pass into the coelom, and then enter the anterior openings of the oviducts. The ova move slowly along the oviduct where they are coated with thin layers of jelly. The ova accumulate in the posterior portion, or uterus, of each oviduct. When actual mating begins the male frog clasps the female in such a manner that his cloacal opening is directly over hers. The ova pass out of the female's body into the surrounding water and concurrently the male sheds sperm over them. The thin, almost invisible, jelly layers surrounding the fertilized ovum now imbibe water and begin to swell, eventually reaching a diameter about three times that of the egg. This jelly is at first sticky and adjacent eggs adhere to one another. As a consequence all of the eggs, which may number more than a thousand, stick together and form a large globular mass in which the embryos develop, each in its own jelly envelopes.

Meiosis and fertilization. Complex and important internal events have been occurring during this entire period (III, pp. 625–635). The ovarian egg has a very large nucleus, the germinal vesicle. (It is interesting to note the antiquity of some of the terms used in describing development. A

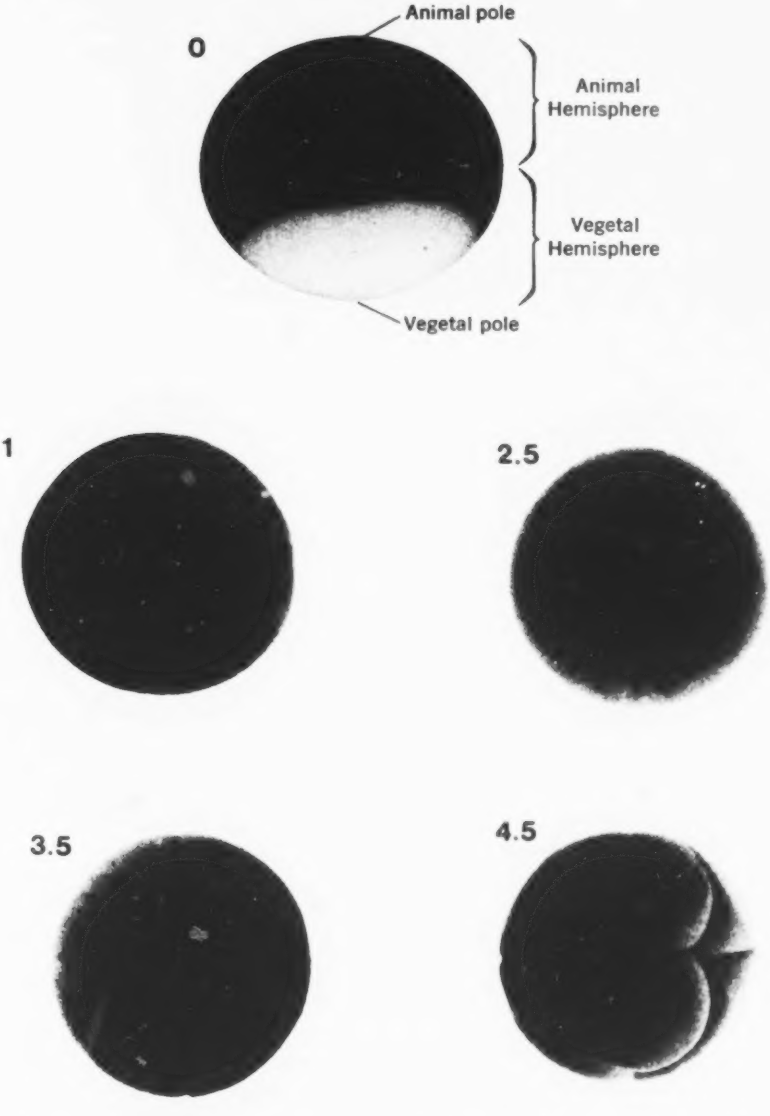

FIG. 17. Development of the frog's egg. Fertilization to eight cells. The 0-hour embryo is in side view; the other embryos are shown looking down on the animal hemisphere. The numbers to the upper left of each embryo in Figures 17–23 are the hours after fertilization at 20°C.

large spherical object was seen in ovarian eggs before it was realized that it was the nucleus. Since it occurred in the "germ" it was named the "germinal vesicle.") When the ova start to break out of their follicles in the ovary, meiosis begins. This involves the dissolution of the nuclear membrane. The first meiotic division occurs by the time the ovum has reached the upper portion of the oviduct and the first polar body is given off at that time. Metaphase of the second meiotic division is reached by the

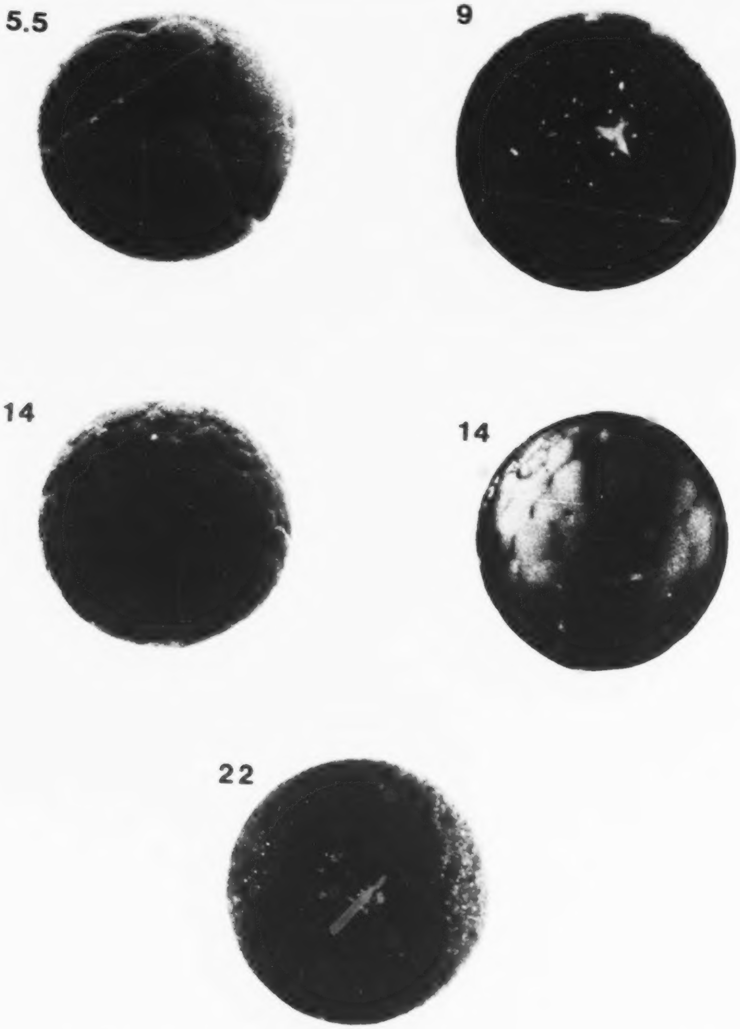

FIG. 18. Development of the frog's egg. **Sixteen cells to late blastula.** Animal hemisphere views except for the rightmost 14-hour embryo, which shows the vegetal hemisphere.

time the ova are in the uterus. Further nuclear changes are blocked at that stage.

A single sperm enters the ovum. Its head contains the paternal nucleus with the monoploid number of 13 chromosomes. A centriole is immediately behind the sperm head. It will become part of the first mitotic spindle. The entrance of the sperm removes the meiotic block and the second polar body is extruded in about a half hour. The maternal pronucleus now has the monoploid number of chromosomes. The two pronuclei move toward the upper center of the egg and there unite, restoring the diploid number of 26 chromosomes.

The uncleaved zygote. The just-fertilized ovum is a sphere approximately 1.7 mm in diameter. Somewhat more than half of the embryo, the animal hemisphere, is a dark chocolate-brown and the remainder, the

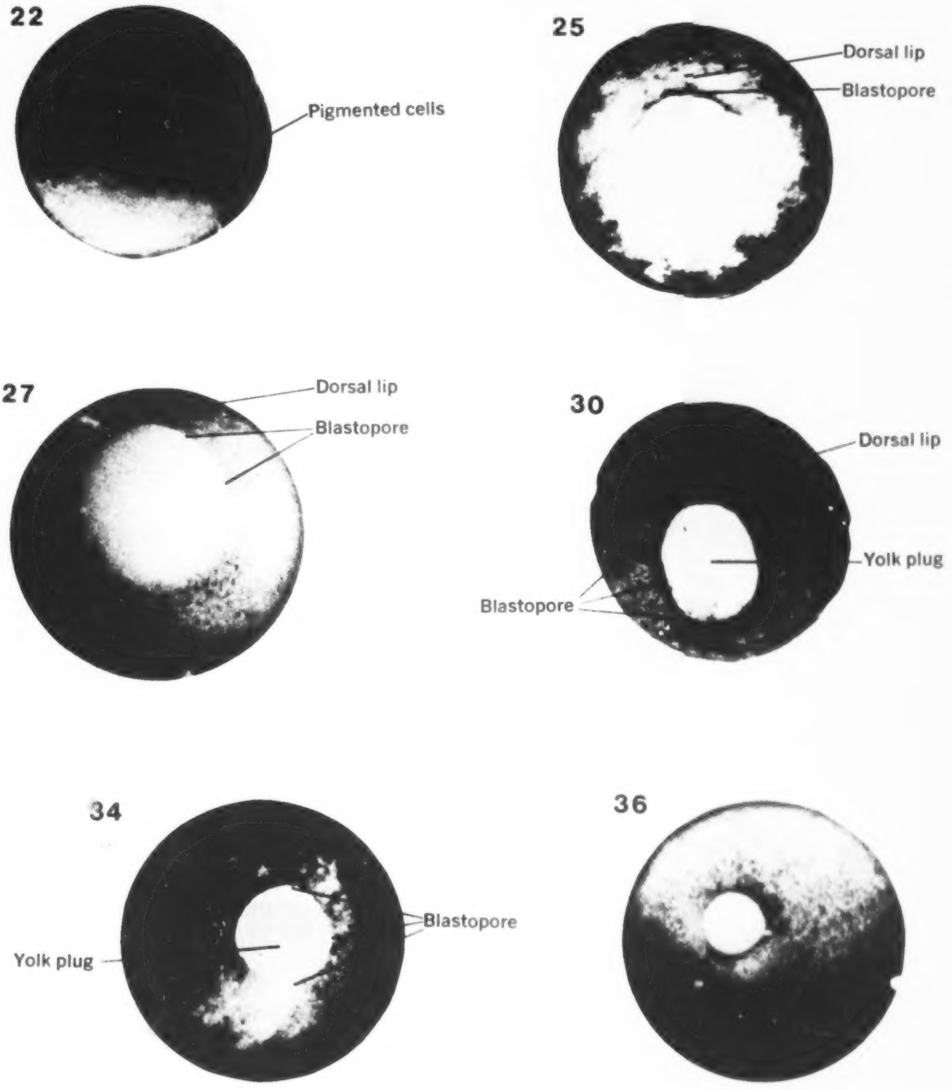
22
Pigmented cells
25
Dorsal lip
Blastopore
27
Dorsal lip
Blastopore
30
Dorsal lip
Yolk plug
Blastopore
34
Yolk plug
Blastopore
36

FIG. 19. Development of the frog's egg. Gastrulation. The 22-hour embryo is in side view; the others are ventral views.

vegetal hemisphere, is almost white (Fig. 17, 0 hours). The animal pole is in the center of the animal hemisphere. It is the site of polar body formation. The vegetal pole is 180° from the animal pole and in the center of the vegetal hemisphere. When the eggs are first deposited they are arranged at random in relation to their polarity. In about an hour after fertilization the swelling of the membranes surrounding the embryo leaves a space between the egg surface and the membranes. This allows the embryo to rotate and the heavier part, the yolky vegetal hemisphere, becomes bottommost. At this time when one examines a mass of eggs

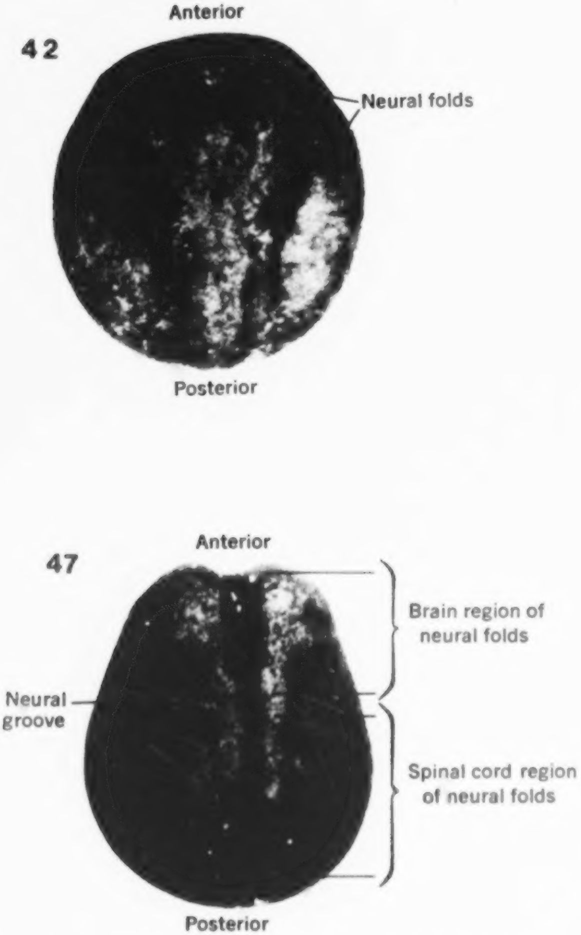
42
Anterior
Neural folds
Posterior
47
Anterior
Brain region of neural folds
Neural groove
Spinal cord region of neural folds
Posterior

FIG. 20. Development of the frog's egg. Early and middle neurula.

from above the dark animal hemispheres will be all that is seen (Fig. 17, 1 hour) as all of the eggs will have rotated. If the egg mass is turned over, all one sees will be the white vegetal hemispheres. Quite quickly, however, the embryos rotate so the animal hemispheres are again uppermost.

Cleavage. (This is another antique term. The "cleaving" of the eggs into smaller parts was observed long before there was any concept of cells or cell division. It was most puzzling to early observers and it took about two centuries to understand what was going on.)

Two and a half hours after fertilization the first spectacular event that is externally visible occurs. A short groove appears in the animal hemisphere and it gradually lengthens to form the first cleavage furrow. The furrow slowly extends through the embryo until two cells are formed. Internally mitosis had begun before the cleavage furrow appeared. When the chromosomes are in telophase the furrow starts to form.

The second cell division begins at about 3.5 hours. The plane of this cleavage is again vertical and at right angles to the plane of first cleavage. Both cleavages pass through or very near to the animal and

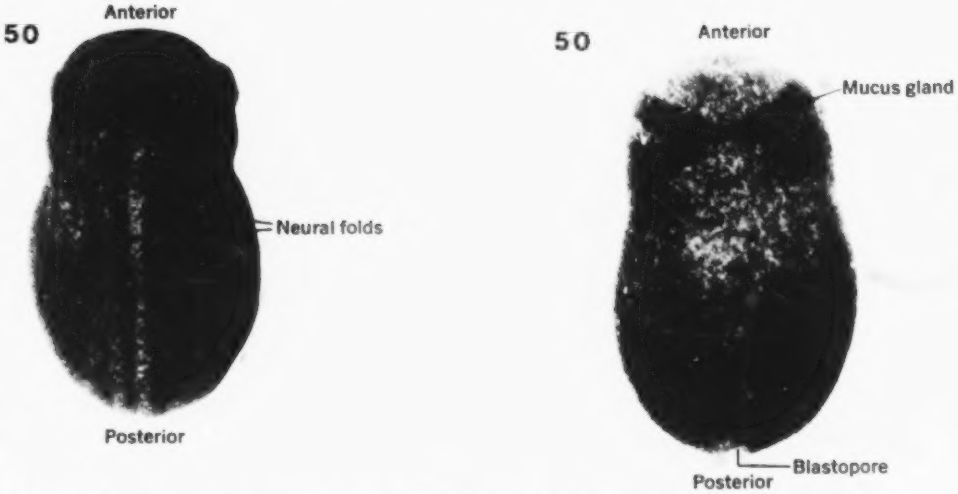

FIG. 21. Development of the frog's egg. Closing neural folds. Dorsal and ventral views of the same embryo.

vegetal poles. The third cell division occurs at about 4.5 hours (Fig. 17). The plane of this cleavage is horizontal. It does not divide the embryo equally, as the plane of cleavage is above the equator. Thus in the photograph one can see the four smaller uppermost cells and beneath them parts of four larger cells. The latter include all of the vegetal hemispheres and the lower part of the animal hemisphere.

When one examines a group of embryos that were fertilized at the same time and kept together, the synchrony of development is awesome. Each of the cleavages starts at almost exactly the same time and at the same place in the animal hemisphere. In fact this synchrony is true of all early development. Each stage is reached at almost the same time in all embryos. It is as though each has an internal clock that was started at the same time and ticks along together with the other clocks. Each must have some sort of biological clock but as yet we know very little about what it is and how it works.

This developmental precision could not have gone unnoticed by embryologists in the 19th century and it would have pressed upon their minds that development, in part at least, must be like a machine. Development seemed precise, uniform, and predictable. When a biological phenomenon has those features one suspects constant cause and effect relationships and of course entertains the hope that they are ascertainable through proper observations and experiments.

The process of cell division continues and soon the intervals between divisions increase. The embryo is divided into smaller and smaller cells and there is no obvious increase in size. We must remember that no food is entering the embryo. Its energy source consists of the yolk granules within each cell. As these are used in metabolism, the dry weight of the embryo decreases. Oxygen diffuses through the jelly layers and enters the embryo and carbon dioxide and some waste materials take the opposite path.

Figure 18 shows this slow decrease in cell size. The 5.5-hour embryo is in the 16-cell stage and by 9 hours there are more than 100 cells. The two photographs in the second row are of a 14-hour embryo. The left one shows the small cells of the animal hemisphere. That embryo was then turned over and the photograph on the right shows the much larger vegetal hemisphere cells. By 22 hours the animal hemisphere cells have become much smaller.

The embryos from 9 to 22 hours are

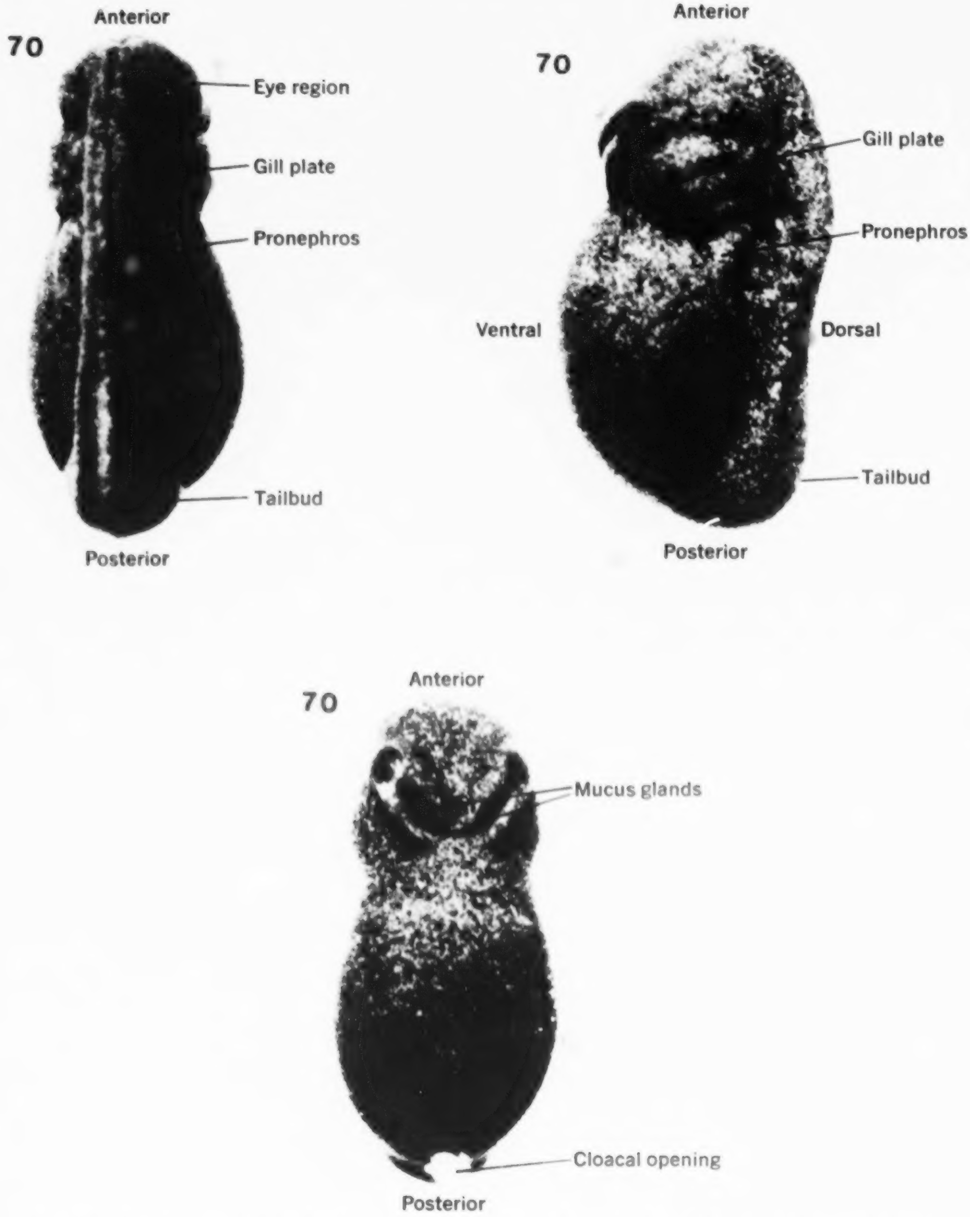
70
Anterior
Eye region
Gill plate
Pronephros
Tailbud
Posterior
70
Anterior
Gill plate
Pronephros
Ventral
Dorsal
Tailbud
Posterior
70
Anterior
Mucus glands
Cloacal opening
Posterior

FIG. 22. Development of the frog's egg. Tailbud embryo shown in dorsal, lateral, and ventral views.

blastulae. The blastula stage is characterized by an internal cavity, the blastocoel, which, when fully formed, occupies most of the interior of the animal hemisphere. More will be said about it later when we consider the internal events of early development.

All of the cells of the late blastula are

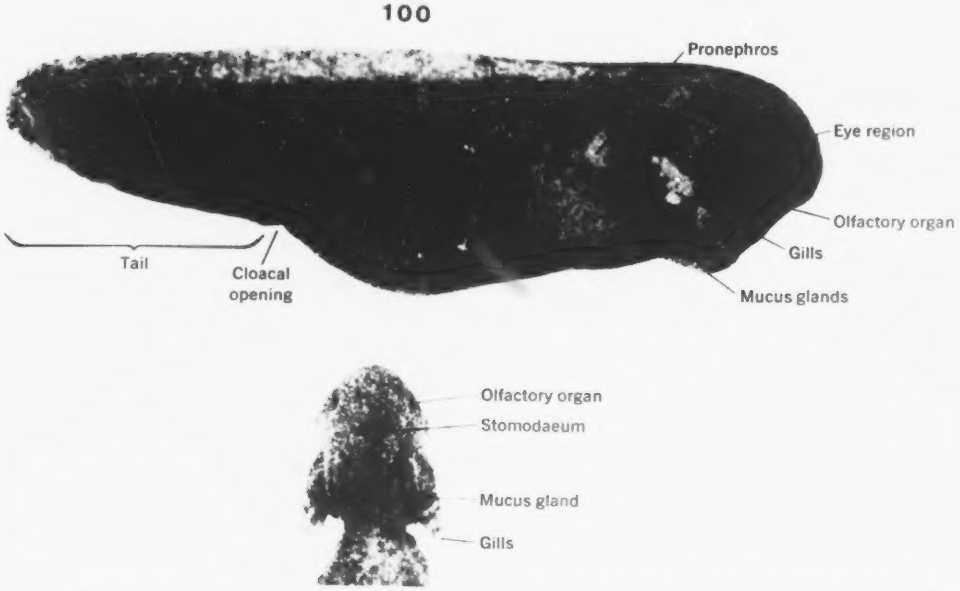

FIG. 23. Development of the frog's egg. Just-hatched larva shown in lateral view and a ventral view of the head.

essentially the same apart from the differences in pigmentation and size. There is a gradient in size that runs from the smallest cells at the animal pole to the largest cells at the vegetal pole. The density of yolk granules follows the same gradient.

Thus the blastula has only a single axis, that extending from animal to vegetal pole. There is no right and no left, that is, no landmark on the sides. If we compare the blastula with the earth, we could recognize a North Pole (animal pole) and a South Pole (vegetal pole). That would enable us to determine the latitude of any position on the blastula but the lack of differences along the sides makes the determination of longitude impossible.

Gastrulation. When the 22-hour blastula is turned over, one observes a narrow groove of pigmented cells in the vegetal hemisphere just below the equator (Fig. 19). By 25 hours this groove has become deeper and extended laterally. The groove itself is the blastopore and its formation marks the beginning of gastrulation. The cells immediately above the blastopore are called the dorsal lip of the blastopore. They will play an extraordinary role in development.

Gastrulation is a process that leads to a complete rearrangement of the cells of the embryo. Many of those on the outside of the blastula will move to the interior. This process of moving in at the lips of the blastopore is called invagination. Figure 13 shows examples of gastrulation in embryos with very little yolk. This process is greatly modified in the frog because of its large, relatively yolky egg but the events are fundamentally the same.

The blastopore of the 25-hour embryo leads into a tiny archenteron. Notice also that the pigmented surface appears to be enlarging. Compare its extent in the 22- and 27-hour embryos. The dark cells of the animal hemisphere are actually moving downward and the lighter cells of the vegetal hemisphere are moving to the interior as the animal hemisphere cells cover them. By 27 hours the lateral lips of the blastopore have extended to the sides and by 30 hours they have finally met to form a 360° blastopore. The area of light-colored cells has become much smaller and they form the yolk plug. Gastrulation continues until, by 36 hours, the animal hemisphere cells have almost overgrown the embryo and the only original vegetal cells seen on the outside are those of the ever-smaller yolk plug. Finally the yolk plug itself moves to the interior and the blastopore is reduced to a tiny slit, marking the end of gastrulation. Now the dark animal hemisphere cells cover the entire surface. The cells have become so small that at moderate magnification they are invisible. The embryo has a superficial resemblance to an uncleaved egg when viewed from the top—it is black and "structureless." You can guess how confusing this stage was to some of the earlier observers.

With the formation of the dorsal lip of the blastopore at 25 hours, we are finally able to specify longitude. Therefore, once gastrulation has begun, we can describe any spot on the surface of the embryo in terms of its latitudinal distance from the animal pole and its longitudinal distance from the dorsal lip. Why one would even wish to do such a thing may not be clear now, but it soon will be.

At the end of gastrulation there is still essentially no obvious cellular differentiation beyond that of the blastula. The diameter of the late gastrula is about the same as that of the uncleaved egg. The rate of metabolism has increased and, since there is still no external source of food, the dry weight is less than before. There are two cavities in the embryo—the vanishing blastocoel and the enlarging archenteron. These are filled with fluid.

Neurulation. The next prominent external change is the beginning of the formation of the nervous system. It is usually surprising to students to find that, as in the frog, their brains actually start developing on the outside of the body. Figures 20 and 21 show the sequence of events. In the 42-hour embryo the neural folds appear as low ridges on the dorsal side of the embryo. These folds are formed as paired structures, extending from each side of the blastopore region anteriorly to where they connect in the region that will become the head. By 47 hours the folds are more elevated and begin to close and by 50 hours the folds touch along their entire length. The folds close in such a manner as to form an internal tube, the neural tube. The walls of the broad anterior portion of the neural folds will become the brain and the walls of the more posterior portion will become the spinal cord. The bore of the neural tube remains even in adult life as the neurocoel.

The embryo at 42 hours is still spherical but it begins to elongate as neurulation proceeds. This growth in length is not the same in all areas. This can be illustrated by what happens in the central nervous system. At 47 hours the lengths of the brain and spinal cord regions of the neural folds are roughly the same. Later in development the spinal cord area will increase in length much more than the brain.

When the 50-hour embryo is turned over (Fig. 21, right) one can see the beginnings of still another structure, the mucus glands. This structure secretes a sticky mucus that enables the larva to attach to various objects.

Tailbud stage. After another day of development there are more external changes (Fig. 22). The elevated ridge along the back contains the brain and spinal cord. Paired bulges in the brain indicate the place where the eyes are forming. Large swellings behind the eye region are the beginnings of the gills. Still farther back a small swelling marks the site where the pronephros is beginning to form. On the ventral side the mucus glands are better developed.

The 100-hour embryo. It is not too difficult to extrapolate from the 70-hour tailbud stage to the embryo of 100 hours (Fig. 23). By 100 hours the embryo has reached the point where the precursors of all its organ systems have formed and some are beginning to function. For example, circulation has begun and close examination shows blood cells moving through the gills. Externally the embryo has begun to resemble a tadpole. The eyes are present as bumps on the side of the head but they are not yet functional—the overlying skin is still deeply pigmented. The olfactory organs are paired pits at the anterior end. Between them is another pit, the stomodaeum. This pit will break through to the primitive alimentary canal, forming the mouth. There is a well-developed tail, that is, a portion of the body posterior to the cloacal opening.

The embryo hatches from its jelly envelopes at about this time. Most of its yolk will have been consumed but, before all the yolk has gone, the young creature will have begun to find and eat food in the pond where it is living.

Thus in a period of about four days, at 20°C, the frog-to-be will have started as a single cell and, with cell division, differentiation and growth, produced a larva with all of its organ systems beginning to form and some already functioning. The epigenetic changes will have occurred with a clock-like precision that continues to awe the observer today as much as it did when amphibian embryos were first studied. This is truly an astonishing phenomenon, all the more impressive to observe because our own development occurs in the almost inaccessible interior of the body—unavailable for easy study.

Although amazing external changes have occurred in the developing embryo, they are not as numerous nor as great as what is happening internally—our next topic.

THE AMPHIBIAN EMBRYO—INTERNAL DEVELOPMENT

In all complex animals the vital organ systems are internal—protected by a skin and often by scales, bone, chitin, feathers, shells, or similar structures. In the vertebrates all of these organ systems develop internally as well. This was a difficult problem for early embryologists since techniques were not available for studying these important internal events. However, by the late 19th century, techniques had been developed for imbedding embryos, after fixation, in paraffin wax and then making thin sections. These could be mounted on glass slides, stained, and studied with a microscope. It even became possible to make serial sections of embryos, *i.e.*, beginning at one end and making thin slices of the entire embryo. The slices were then mounted in order on slides and the end result would be hundreds of slices of the entire embryo. One then had the task of deducing the whole internal structure from these thin sections.

An embryo in serial section is static and cannot provide a complete story of the movements of cells to their final sites where they produce the various structures. One can not determine, for example, how the archenteron forms from looking at slides. Does it involve an invagination of cells from the outside or is it a matter of new cells being formed at the advancing edge of the archenteron?

Consider the events in the early development of the frog (Fig. 19). The changes from 22 to 36 hours can be explained as the downgrowth of the dark-colored animal hemisphere cells over the light-colored vegetal hemisphere cells. Alternatively, the events could be explained equally well by assuming that the light-colored cells slowly become pigmented.

How could one decide between these two hypotheses? Some early experimental embryologists sought an answer by pushing a needle through the jelly membranes and killing some of the cells on the surface of the embryo. One could then trace the movements of the scar for as long as it lasted, which often was not very long. Nevertheless, experiments of this sort made it seem true beyond all reasonable doubt that cells on the outside did move down from the animal hemisphere.

By the 1920s the experimental analysis of the development of the amphibian embryo had reached the stage where it was necessary to know, with a high degree of accuracy, the direction of movement of the various parts of the embryo during gastrulation.

The basic problem was to be able to describe accurately all positions on the embryo and to be able to trace these positions throughout early development. We have seen already that in a late blastula one can determine position only in terms of distance from the animal pole. To return to our analogy with the earth, if we were told only that Philadelphia was 50° from the North Pole, we would have no way of knowing whether it was in Spain, Turkey, Russia, China, or the United States.

It is only when the dorsal lip of the blastopore appears at the onset of gastrulation that, by analogy, we have the zero meridian of Greenwich. The ability to determine distance from the animal pole and from the dorsal lip means that any position on the surface of the early gastrula can be described accurately.

But experimentalists needed to know not only where a given group of cells might be at the onset of gastrulation but where these same cells would be at various times thereafter. Would they be in the same place or would they have moved?

THE FATE MAP

It took a German embryologist, Walther Vogt, many seasons of painstaking observation and experimentation to provide an acceptable answer. The term "seasons" is employed because the amphibian embryos used in his experiments came from breeding adults and the breeding season was restricted to a few weeks during the year.

Vogt sought to define the location in an early gastrula of the cells that would later become the three germ layers: ectoderm, mesoderm, and endoderm. That is, he wished to determine the eventual fate of all of the cells. The results could then be expressed by a map-like diagram of an early gastrula showing where the cells would be in a later embryo. Such a diagram is called a Fate Map.

Vogt found that the cells destined to form each layer occurred together at the onset of gastrulation and that each remained as a unit throughout gastrulation. He found also that, within the limits of his ability to measure position, cells in different embryos behaved in precisely the same manner.

The technique for making the observations necessary for the construction of a fate map is as follows. A layer of wax is put in a small dish and a small pit, about the size of an early gastrula, is made in the surface (Fig. 24, top). Tiny pieces of agar are stained with a variety of vital dyes and placed in the sides of the pit. The jelly membranes are removed from an early gastrula, leaving only the vitelline membrane, and the embryo is pushed into the pit. It is held in place by a tiny piece of bent cover glass.

Some of the dye would diffuse from the agar and stain the cells on the outside of the embryo. Differently colored vital dyes were used, thus allowing individual spots on the embryo to be differently stained. After exposure to the dyes, the embryo would be removed from the pit and a drawing immediately made of the position of the colored spots. At frequent intervals thereafter the same embryo was studied and sketched. Some of the colored spots were invaginated. In these cases it was necessary to dissect the embryo and ascertain the position of each spot.

The three diagrams at the bottom of Figure 24 show one of Vogt's experiments. Eight colored spots, 1 through 8, were placed on the embryo along the meridian that passes through the animal pole and the dorsal lip of the blastopore. A short time later, Vogt found that spot 7 had moved to the interior and that spot 6 was now the dorsal lip (diagram *a*). In the middle gastrula, *b*, only spots 1–4 remain on the outside. They did not remain in that position, however, but became stretched to cover a much larger portion of the surface of the late gastrula, as shown in *c*.

After performing hundreds of experiments of this sort, Vogt was able to prepare a fate map (Fig. 25) of the early gastrula of the European toad, *Bombinator*. He studied other amphibians as well. He found that the cells that form the three germ layers were laid out on the surface of the early gastrula as shown.

This is a complex diagram and is difficult to understand when first seen. However, it is a valuable conceptual scheme for understanding how the germ layers, and later the organ systems, are formed. It will be a point of reference in describing further development. Some general introductory remarks about Figure 25 may be useful.

The presumptive ectoderm, that is, the cells that will form the ectoderm later in development, occupies nearly all of the animal hemisphere. Two main subdivisions are delimited in Figure 25: the presumptive neural tube, an area consisting of those cells that will eventually form mainly the brain, spinal cord, and optic cup; and the presumptive epidermis, which occupies

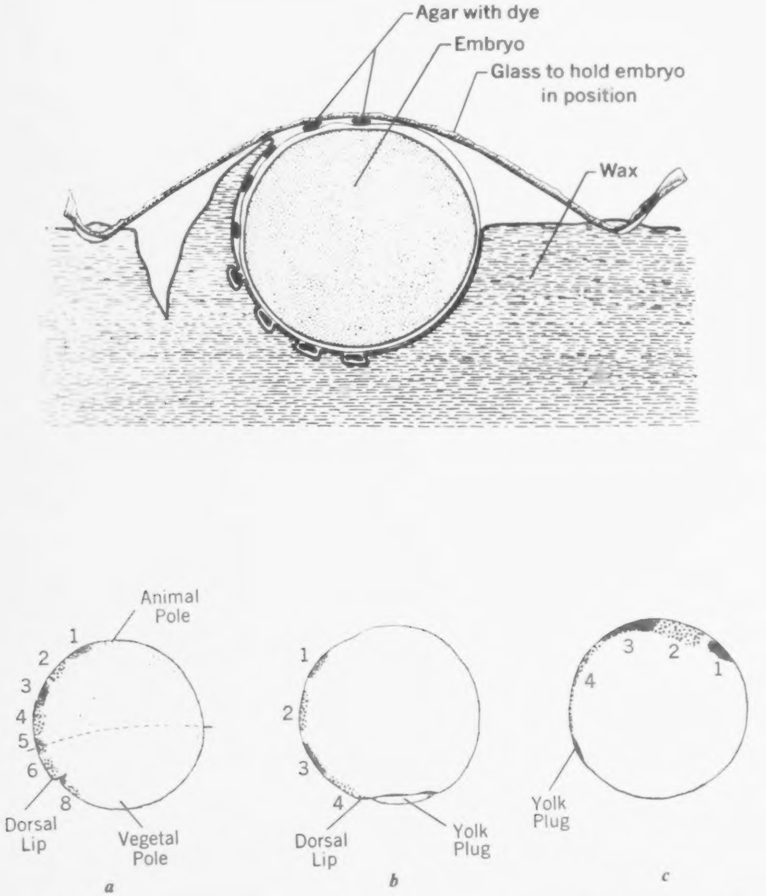

FIG. 24. Vogt's technique for staining embryos is shown in the upper figure. The three lower figures show one of the experiments. The vitally stained spots have been given numbers. *a* is an early gastrula with the dorsal lip below 6; spot 7 had already invaginated. *b* is a mid gastrula and spots 5, 6, 7, and 8 have invaginated. *c* is a late gastrula and only spots 1, 2, 3, and 4 remain on the outside. Compared to their positions in *a*, they have spread considerably. Note the positions of all these spots with the fate map in Figure 25. (From Vogt, 1925 and 1929.)

about a quarter of the surface of the early gastrula and will eventually spread to form the entire epidermis covering the embryo and later the adult.

The presumptive mesoderm forms a band of cells surrounding the embryo in the equatorial region. It, too, consists of two main areas: the cells immediately above the dorsal lip will form the notochord; the remainder of the presumptive mesoderm will form the muscular, skeletal, circulatory, reproductive, and excretory systems as well as connective tissue and coelomic epithelia.

The presumptive endoderm occupies much of the vegetal hemisphere. Its cells will form the lining of the alimentary canal and structures derived from it such as the liver, pancreas, and bladder.

The presumptive mesodermal and presumptive endodermal cells that are on the outside of the early gastrula will all be invaginated to the interior during gastrulation. The division between what goes in

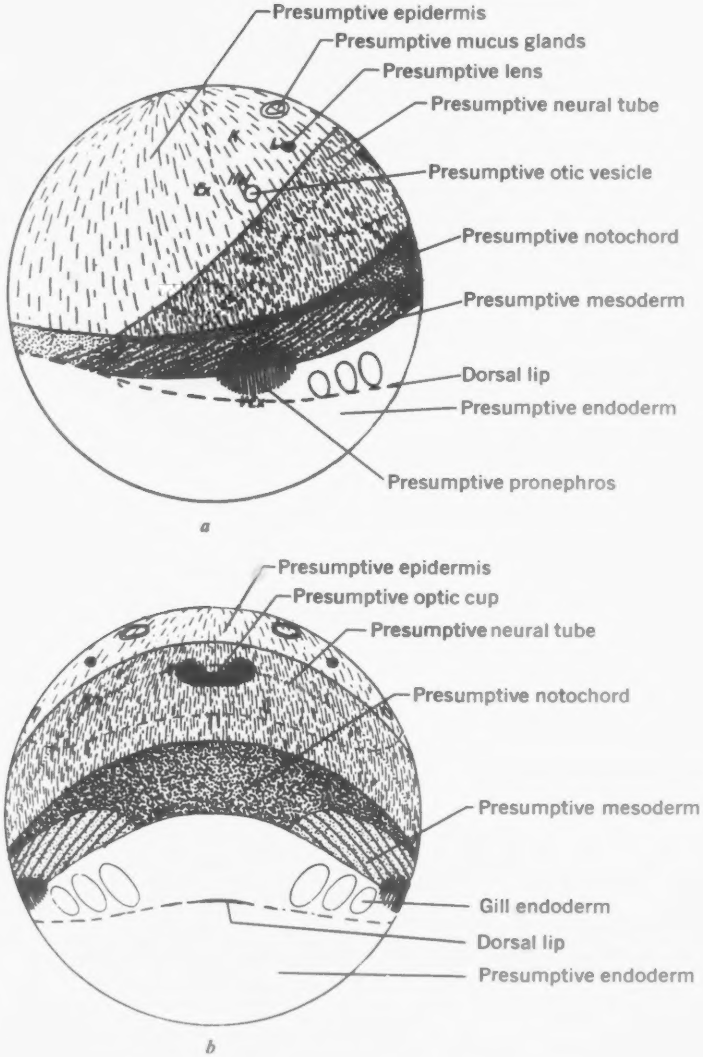
Presumptive epidermis
Presumptive mucus glands
Presumptive lens
Presumptive neural tube
Presumptive otic vesicle
Presumptive notochord
Presumptive mesoderm
Dorsal lip
Presumptive endoderm
Presumptive pronephros
a
Presumptive epidermis
Presumptive optic cup
Presumptive neural tube
Presumptive notochord
Presumptive mesoderm
Gill endoderm
Dorsal lip
Presumptive endoderm
b

FIG. 25. Vogt's Fate Map for *Bombinator* (Vogt, 1929).

and what stays out is shown in Figure 25 by the line that separates the presumptive ectodermal areas from the presumptive mesoderm. Note also the dotted line that starts at the dorsal lip and extends around the embryo. That is the line that marks the region of invagination.

Yes, these events are complex—but wait. When we look at diagrams of embryos it will become clearer. Vogt's discoveries are important for us since they are basic to understanding the experiments from Roux to Spemann that have thrown so much light on the underlying causes of differentiation.

CELL MOVEMENTS DURING GASTRULATION

Before gastrulation cell division divides the embryo into many cells, and a small cavity, the blastocoel, appears in the animal hemisphere. In the 12-hour blastula the blastocoel is still small and the surrounding cells are many layers thick (Fig. 26).

By 22 hours (Fig. 27) the blastocoel occupies most of the animal hemisphere and its roof is only a few cells thick. The pigmented cells that foreshadow the site of the dorsal lip have appeared by this time, though the photograph does not show them. It is now possible to use Vogt's fate map to demarcate the positions of the presumptive germ layers. Using the fate map shown in side view (Fig. 25, top), an imaginary slice of the embryo has been made through the meridian that includes the animal and vegetal poles and the dorsal lip. This slice is diagrammed in Figure 27*b*. Most of the blastocoel roof consists of presumptive epidermis and anterior to this are the presumptive neural tube and notochord. The dorsal lip will form at about 4 o'clock. The presumptive notochord cells are in the area above the dorsal lip and they are continuous with a band of the other mesodermal cells that extends entirely around the embryo. Essentially all of the vegetal hemisphere is presumptive endoderm.

Further movements of the cells are shown in Figures 28 through 32, which should be studied in relation to the whole embryos shown in Figures 18 through 22.

The archenteron of the 30-hour embryo (Fig. 28) is a thin cavity opening to the outside through the blastopore. It appears to be pushing ahead of it a wall of cells that encroach upon the blastocoel. The growth of the archenteron is rapid and by 34 hours (Fig. 29) it has almost reached the anterior end and by 36 hours it has done so (Fig. 30). Its leading end continues to push anteriorly, then ventrally, and finally posteriorly, ending in a slight bulge that will form the liver diverticulum—in the 47-hour neurula (Fig. 31). This process continues and by 55 hours the archenteron begins to look more like a tube and less like a huge cavity (Fig. 32). The blastocoel, while still present at 47 hours (Fig. 31) is soon obliterated.

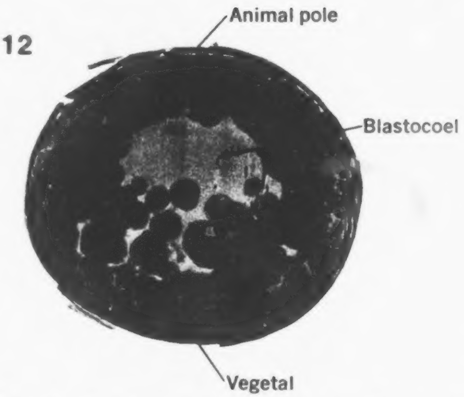

FIG. 26. Development of the frog's egg. Cross section of an early blastula. The numbers to the upper left of the embryos in Figures 26–34 indicate the hours after fertilization.

The archenteron continues to be open to the outside through the blastopore. (This opening does not show in all of the photographs of the embryos.) The blastopore will close eventually and shortly thereafter the anus will break through near the place where the blastopore closed. The mouth will break through at the anterior end of the archenteron.

In the 30-hour embryo (Fig. 28) the presumptive notochord cells are moving inward to form the dorsal wall of the archenteron. This ingression continues until the entire area is inside by about 36 hours and it almost reaches the anterior end of the embryo in the 55-hour late neurula (Fig. 32). The rearrangement of the presumptive notochord area involves a considerable change in shape. In the fate maps of Figure 25 the presumptive notochord cells appear as a band extending across the embryo. During gastrulation these cells move to the mid-line and are stretched in an anterior direction.

The presumptive neural tube area undergoes a similar change in shape. In the fate maps of Figure 25 this area also extends across the embryo. Again the gastrulation movements change the long axis to anterior–posterior. In the 47-hour embryo (Fig. 31) a portion of the neural folds shows at the anterior end. If this seems to be a strange place, look at the whole embryo in Figure 20. A slice in the mid-line (sagittal section) would go between the two neural

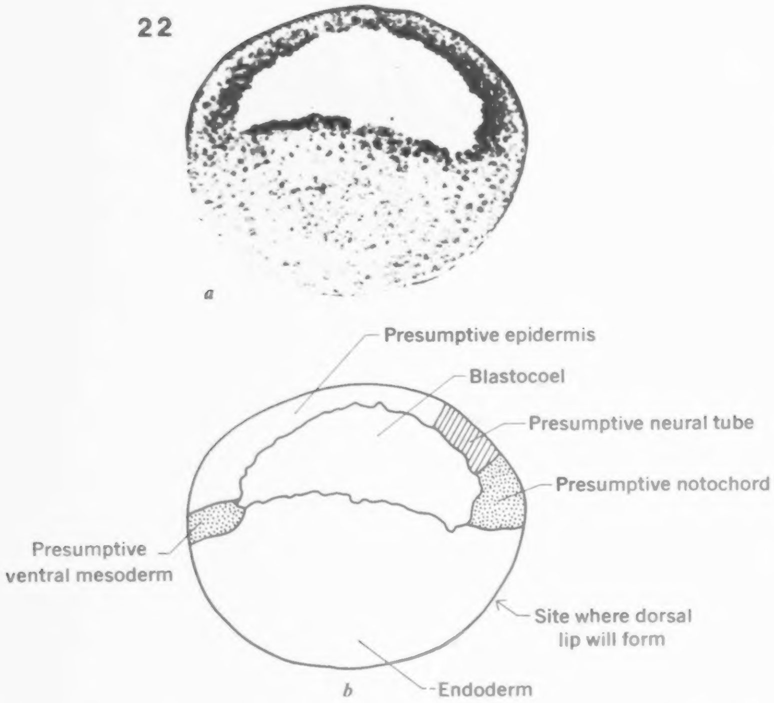

FIG. 27. Development of the frog's egg. Cross section and interpretative diagram of a late blastula.

folds and cut them only at the very anterior end where they are joined. By 55 hours (Fig. 32), however, the folds have closed and then a mid-line section will cut through the neural tube.

Embryos are three dimensional and the two dimensional longitudinal mid-line sections of Figures 27 through 32 tell us nothing of what is occurring on the sides of the embryo. It is necessary to use cross sections, that is, slices across the long axis of the body, for a better understanding of the embryo's structure.

Figure 33 shows what the 47- and 50-hour embryos look like when sectioned at right angles to the plane of the previous sections. The neural folds are about to close at 47 hours. The notochord is in the dorsal mid-line below the neural folds. To either side of the notochord the mesoderm extends a short distance. The huge archenteron is surrounded by endodermal cells.

The 50-hour embryo shows the final and fundamental distribution of the germ layers of a vertebrate embryo. The ectoderm has formed the neural tube and the epidermis that surrounds the embryo. The middle layer is mesoderm. It consists of the notochord on the mid-line beneath the neural tube flanked by the lateral mesoderm, which by this time extends as a thin layer entirely around the body. The inner layer, the endoderm, surrounds the archenteron.

By a day and a half later, at 80 hours (Fig. 34), there have been considerable further developments. The cross section of the head shows that the neural tube has enlarged to form the brain and from its ventro-lateral walls the optic cups have grown out. The optic cups will form the retina, the light-sensitive portion of the eye. The epidermis adjacent to the optic cup forms the lens. The notochord does not appear in this very anterior section. Figure 32 shows why.

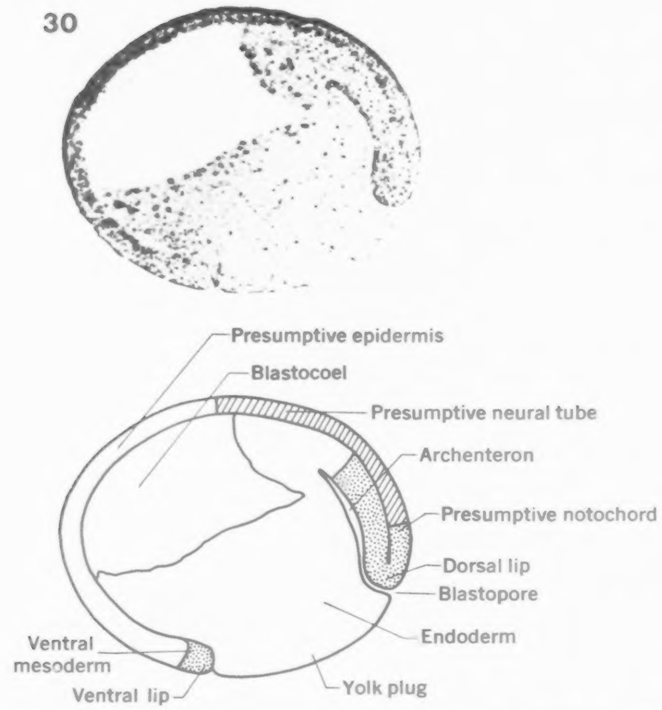

FIG. 28. Development of the frog's egg. Sagittal section and interpretative diagram of a 30-hour gastrula.

A section made in the heart region of the same embryo shows additional structures. The neural tube at this level is the hindbrain, which will form the medulla. Otic vesicles have been formed from the outer ectoderm and will differentiate into the inner ear. The heart is forming as a delicate tube beneath the archenteron. The cavity surrounding it is the pericardium, which is part of the coelom.

The cross section of the middle of the body shows an additional structure—the pronephros. It is the first stage in the development of the excretory system. The mesoderm on either side of the nerve tube and notochord has differentiated into the myotomes or somites, which will form the voluntary muscles and parts of the skeleton. The more ventral mesoderm will eventually split along its length and the cavity so formed will be the coelom.

This brief survey of early development of the amphibian embryo will provide a basis for understanding the experiments that, beginning in the 1850s, sought to explain differentiation. Now that we have surveyed *what* happens, we can try to understand *how* it happens.

THE DAWN OF EXPERIMENTAL EMBRYOLOGY

The books that students read and the university lectures they attend cannot fail to leave the impression of the inevitability of progress in science. Practitioners of science know better. Every important discovery is a rare event that is preceded by a series of failures.

A lesson that can be learned from the study of progress in embryology is how slow progress has been and how exceedingly difficult it has been to understand the underlying mechanisms that transform the relatively simple zygote into a complex adult. In fact, there was no effective experimentation from Aristotle in 4th century B.C.

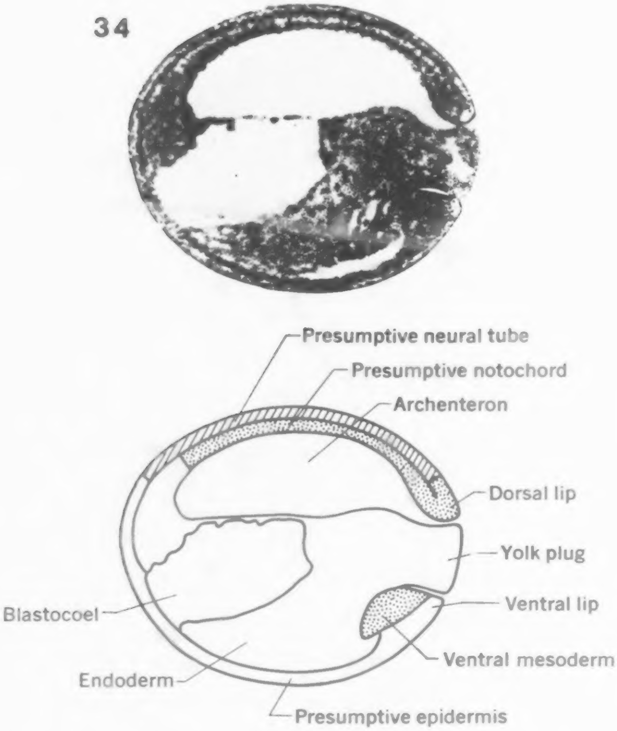

FIG. 29. Development of the frog's egg. Sagittal section and interpretative diagram of a 34-hour gastrula.

to George Newport in mid-19th century Victorian England. Why these long centuries of stasis? There is a simple answer: no one knew how to ask a useful question.

Once again it is helpful to students if they are asked, now that our long survey of descriptive embryology has been concluded, "How would you go about seeking ways to understand the mechanisms of development?" "What are some of the important problems that should be solved?" The answers are far from obvious.

GEORGE NEWPORT

Horder *et al.* (1986, p. xix) credit George Newport (1802–1854) with performing "the first experiment on embryos: point of sperm entry determines axis of developing embryo." There had been many sorts of crude experiments on embryos long before Newport but we have in him a person with a clear notion of what he wished to do and great skill in making observations and performing experiments. Experimental embryology did not begin with Newport but with him it most surely took a quantum leap.

Newport was primarily interested in fertilization and the factors influencing it (1851, 1853, 1854). First he studied the ovarian eggs of frogs, noted the breakdown of the germinal vesicle, described the passage of the ova through the body cavity into the oviducts, and their storage in the uterus. He found that the jelly layers deposited while the ova passed through the oviducts were necessary for fertilization. He stripped semen from the males and carried out many experiments on the relation of sperm concentration and motility to fertilization as he tried various temperatures and chemical solutions to find their effects on fertilization.

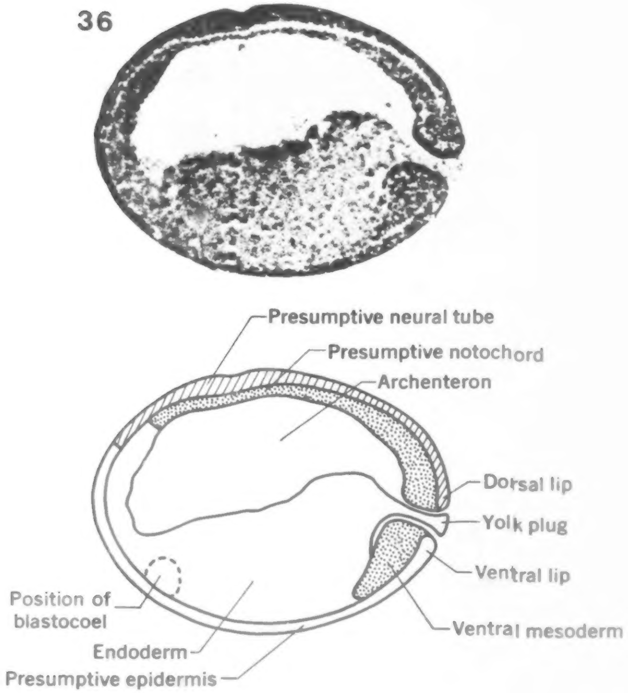

FIG. 30. Development of the frog's egg. Sagittal section and interpretative diagram of a 36-hour gastrula.

At first he did not accept other reports or his own observations as indicating that the sperm actually enters the ovum. Eventually he did so and he is now generally regarded as the first person to offer conclusive proof of this fundamental event.

The fertilized egg of a frog is an enormous sphere compared to sperm. Therefore the chance of observing sperm penetration is slight since one would be searching a huge surface (as it would appear under a microscope) for a tiny event. Newport successfully solved this problem by controlling the point of sperm entry. He prepared a sperm suspension and then dipped the point of a pin into it. The pin was then touched gently to the jelly membranes as he looked through the microscope. In order to facilitate these observations,

> I employed a glass cell to contain the egg whilst it was examined, with the view of keeping it in one position, and preventing the movement derived from accidental causes: it is made of a section of a piece of barometer tube, from one-eighth to one-fourth of an inch deep and three lines [a "line" is 2.2 mm, or $\frac{1}{12}$ of an inch] in diameter in the clear, which is cemented on a plate of glass of convenient size. This piece of apparatus, which I name a *tube-cell,* is of a size sufficient to contain only a single egg after its covering is fully expanded. For the purpose of making an observation, the egg is to be placed in the centre of the cell, immediately after removal from the body of the frog, and before it has come into contact with any fluid; by this proceeding the gelatinous envelopes adhere so firmly to the glass as to render the egg almost or quite immoveable, when the jelly expands on the subsequent addition of water. In order that the proper focal distance of high magnifying powers may be obtained, I commonly use a cell which allows the object-glass [= objective] to

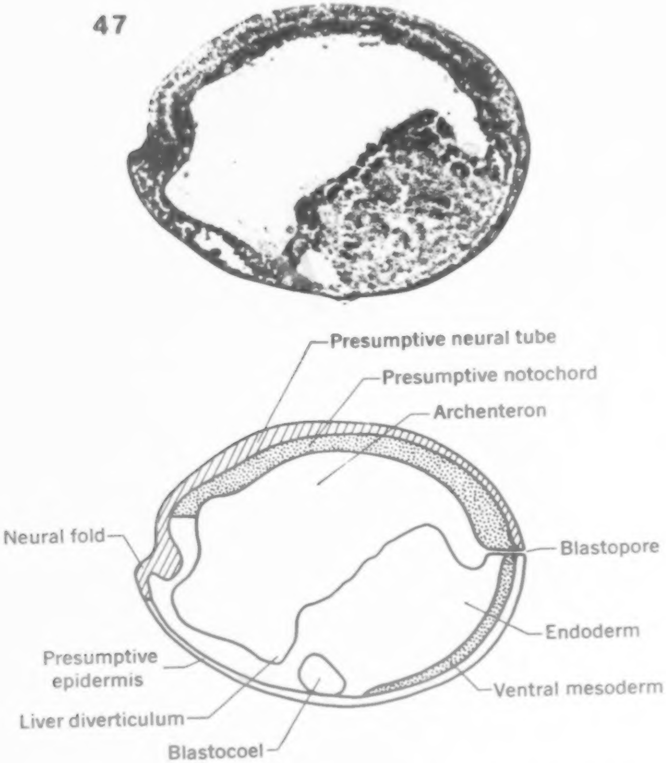
47
Presumptive neural tube
Presumptive notochord
Archenteron
Neural fold
Blastopore
Endoderm
Presumptive epidermis
Ventral mesoderm
Liver diverticulum
Blastocoel

FIG. 31. Development of the frog's egg. Sagittal section and interpretative diagram of a 47-hour neurula.

> be immersed in the fluid. As this cell admits light on every side, it is well adapted for viewing the penetration of the spermatozoon into the egg envelopes . . . (1854, p. 230).

Newport also made a *cistern box*, with flat sides so that he could study eggs from the side—the curved glass of the tube-cell gave considerable distortion.

The ability to immobilize the embryos permitted Newport to make some very important observations about the polarity of the embryo.

> *On the correspondence of the primary cleft of the Yelk with the axis of the future Embryo.*
>
> I have been long aware that the axis of the embryo was in the line of the first cleft [cleavage] of the yelk, but my endeavour to show this was not always satisfactory, in consequence of the difficulty of making the egg keep in a given position, whilst it was free to move; but since I have employed the tube-cell I have obtained the desired evidence with great ease. The results of the following observations will support my statement.
>
> *Obs.* 1.—I took an egg that had just divided for the first time, and placed it in a glass cell only sufficiently large to contain it when the jelly was fully expanded, and filled the cell with water. The dorsal surface turned uppermost, as usual, consequently I had under my eye the whole surface; and could watch the changes with the microscope. I marked the plate of glass supporting the cell with a line parallel to the primary cleft of the yelk, and indicated the position of the

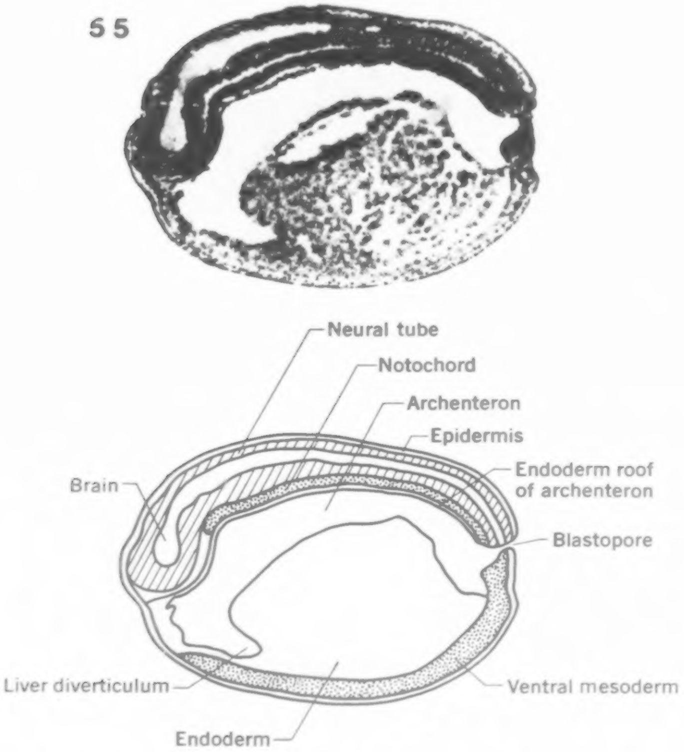
55
Neural tube
Notochord
Archenteron
Epidermis
Endoderm roof of archenteron
Brain
Blastopore
Liver diverticulum
Ventral mesoderm
Endoderm

FIG. 32. Development of the frog's egg. Sagittal section and interpretative diagram of a 55-hour neurula.

ends of the sulcus [furrow] by other marks. The whole was placed in a temperature of 60° Fahr.

At the time of the closing-in of the dorsal laminae [neural folds], I found the correspondence between the axis of the embryo and the line of the first cleft to be exact . . . .

Obs. 2.—Nine eggs were put in separate cells on March 11th, and when segmen-

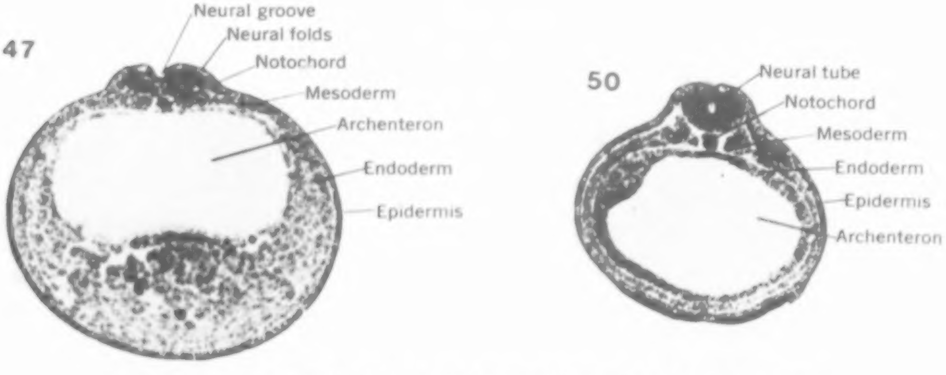
47
Neural groove
Neural folds
Notochord
Mesoderm
Archenteron
Endoderm
Epidermis
50
Neural tube
Notochord
Mesoderm
Endoderm
Epidermis
Archenteron

FIG. 33. Development of the frog's egg. Cross sections of a mid and late neurula.

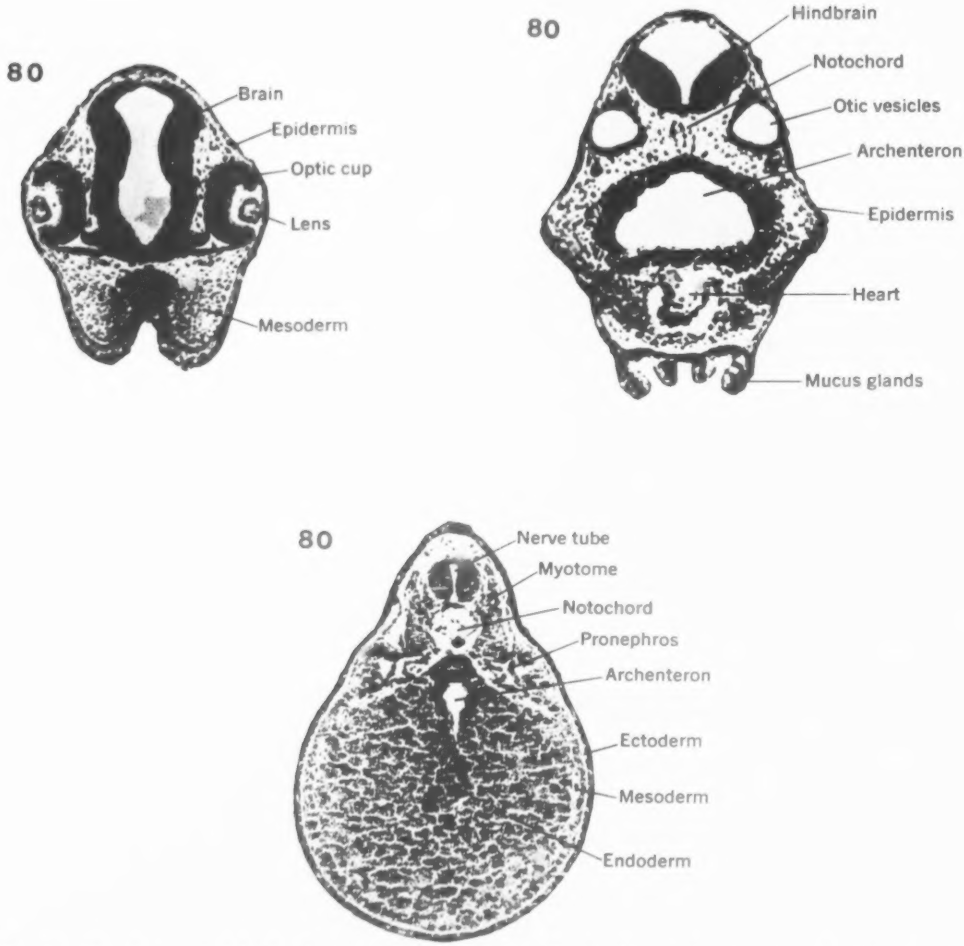
80
Brain
Epidermis
Optic cup
Lens
Mesoderm
80
Hindbrain
Notochord
Otic vesicles
Archenteron
Epidermis
Heart
Mucus glands
80
Nerve tube
Myotome
Notochord
Pronephros
Archenteron
Ectoderm
Mesoderm
Endoderm

FIG. 34. Development of the frog's egg. Cross sections of a tailbud embryo in the eye, ear, and trunk regions.

> tation began, the line of the first cleft was carefully marked on the glass in the manner before explained. One of the eggs was abortive . . . .
>
> March 14. In each of the eight instances the axis of the body is more or less precisely in the line of the sulcus: thus in five it was in the exact line, in one about five degrees to the left, in another about three degrees to the left, and in the remaining one more to the left of the given line (1854, pp. 241–242).

Now comes the remarkable observation that makes it most appropriate to recognize Newport as the first experimental embryologist.

> *On the power of the Spermatozoon to influence in artificial impregnation the direction of the first cleft of the Yelk.*
>
> In connection with the influence of the spermatozoon on the egg, I determined to try whether the artificial application of that body to different parts of the egg's surface could affect the position of the first cleft of the yelk.
>
> *Obs.* 1.—Several eggs were placed, March

> 29, in separate tube-cells, with each turned on its side so that both the dark and white surface were exposed. Very recent spermatic fluid was then applied, by means of a pin's head, to the lower part of the dark surface, and the cell was carefully marked close to the spot, to show where the egg was touched .... April 3. Each egg has formed an embryo, and in each instance with the head to the side of the egg touched.
>
> *Obs.* 2.—Four eggs were placed in separate cells as before, and only two became fruitful. In one the primary cleft was in the precise line of the spot touched, although the egg subsequently diverged to the left; and the head corresponded to the part fecundated. In the other the cleft was about ten degrees to the left of the part impregnated, and the head was also turned to the part touched with fluid.
>
> *Obs.* 3.—Four other eggs were taken, but two of them were sterile; and in the development of one the head deviated remarkably from the usual position. The first cleft in one (*a*) was about six degrees to the right; and in the other (*b*) about five degrees to the left of the point touched. Both formed embryos: in one (*a*) the head was at the end of the cleft nearest the point touched, but in the other (*b*) at the end furthest from the same point. The peculiarity in the last experiment I cannot explain; possibly there might be some want of precision in conducting it.
>
> Similar experiments were repeated four other times, and the results showed that the first cleft of the yelk is in a line with the point of the egg artificially impregnated, and that the head of the young frog is turned toward the same point (1854, pp. 242–243).

Newport was always careful to mention any deviation from what he had come to expect, but he states that the deviation never exceeded 15°. Today it seems remarkable that the results are so consistent. After all, one might not expect the sperm to enter the ovum at exactly the spot touched with the sperm-laden pin.

That ended Newport's career—he became ill after a collecting trip in a swampy area near London and died. In fact the 1854 paper was completed after his death by a friend.

One can only speculate how Newport would have built on this fundamental discovery to continue his experiments. Not only had he established a causal relationship between the entrance point of the sperm, the plane of first cleavage, and the primary axis of the embryo, but he could control that relationship. This ability to control development in such a basic way made possible the experimental analysis of differentiation. One could begin to ask meaningful questions and have some hope of being able to answer them.

It is important to note that a breakthrough in experimental science frequently comes as a result of observations having little to do with the problem being explored. Newport was initially mainly interested in fertilization. The tube-cells that he constructed to observe sperm penetration better held the embryo in a fixed position. This same experimental setup proved to be valuable in another way—to make possible his observations associating the entrance point of the sperm, first cleavage and the embryonic axis.

Experimental embryology was underway. Well, not quite. Newport's remarkable discoveries were not to be extended for several decades. Darwin was shortly to capture the interest of embryologists and experimentation was to receive little attention. In fact, the data that were essential for the further analysis of development were to come from the study of cells, not from the study of embryos.

DEVELOPMENT, HEREDITY, AND THE CHROMOSOMES

The greatest puzzle of all in the mid-1800s was, How can the fertilized ova of two species, which may seem identical, develop into two adults that differ greatly from one another? Whatever the nature of heredity might be, surely it must be fundamental in determining the pattern of development. With the passage of each decade the possibilities of spontaneous generation seemed ever less likely. It seemed more and more probable that living organisms could come only from living organisms and that cells could come only from cells. Thus, whatever its nature, inheritance must be associated with the dividing cells of the developing individual and with the cells of reproduction—ova and sperm.

Schwann's and Schleiden's paradigm of the Cell Theory, plus the great improvements in methods for studying cells, resulted in a burst of cell research beginning in the 1870s and lasting to the turn of the century. There is a lengthy discussion of the results in last year's Essay (III, pp. 609–639) so there is no need to repeat the details here. These are the general conclusions.

1. The reproduction of somatic cells results in the formation of daughter cells identical with each other and with the parent cell.

2. That means that the structures of cells must also reproduce or be synthesized. For both the cell and its parts there must be a doubling and then division.

3. Some cell structures appear to be passively allocated to the daughter cells.

4. On the other hand chromosomes appear to be divided by a complex and precise mechanism—mitosis. Each chromosome replicates and then one daughter chromosome goes into each daughter cell.

5. In some species, at least, the number of chromosomes appears to be constant.

6. This number remains constant from generation to generation: hence, there must be some mechanism for maintaining this constancy. The mechanism was found to consist of two modified nuclear divisions—meiosis.

7. Meiosis in males was found to occur just prior to the formation of mature sperm. In females it may begin either at the time of ovulation or just after fertilization. Meiosis consists of two divisions the result being a halving of the number of chromosomes. Thus, after meiosis has been completed, both ovum and sperm have the monoploid number of chromosomes.

8. The fusion of a monoploid female pronucleus and a monoploid male pronucleus at fertilization restores the diploid number in the new individual.

9. Although there were many variations in different kinds of animals, the basic patterns of mitosis and meiosis were found to be remarkably constant throughout the animal kingdom.

10. These complex, remarkable, and nearly universal mechanisms for maintaining nuclear and chromosomal constancy, plus a few crude experiments, made probable the hypothesis that the nucleus, and more specifically the chromosomes, play an important role in heredity.

A caution should be entered at this point. The concept of the chromosomes as the physical basis of inheritance is so firmly embedded in the way we think today that we tend to forget that a hundred years ago that was not the case. Some biologists did regard that notion as a probable hypothesis but many still treated heredity as an abstract idea and not as a phenomenon closely associated with known structures of the cell. Other biologists at that time thought more in terms of the idioplasm hypothesis of Carl von Nägeli, who proposed that the physical basis of inheritance consisted of an invisible network that extended throughout all cells (III, p. 636). Some thought of the idioplasm as Darwin's gemmules (III, pp. 596–605) linked together in an organized structure. The hypotheses of chromosomes *versus* idioplasm as the substance of inheritance were not mutually exclusive, since the idioplasm was thought to spread through the nucleus as well.

E. B. WILSON: STATING THE PROBLEM

In the 1870s when the experimental analysis of development began to attract more investigators, there was a need to define the problem. What it was has been said well by E. B. Wilson. Although the following quotation was written later (1900) he expressed a point of view that would have been much the same two decades earlier.

> Every discussion of inheritance and development must take as its point of departure the fact that the germ is a single cell similar in its essential nature to

> any one of the tissue-cells of which the body is composed. That a cell can carry with it the sum total of the heritage of the species, that it can in the course of a few days or weeks give rise to a mollusk or a man, is the greatest marvel of biological science. In attempting to analyze the problems that it involves, we must from the onset hold fast to the fact, on which Huxley insisted, that the wonderful formative energy of the germ is not impressed upon it from without, but is inherent in the egg as a heritage from the parental life of which it was originally a part. The development of the embryo is nothing new. It involves no breach of continuity, and is but a continuation of the vital processes going on in the parental body. What gives development its marvelous character is the rapidity with which it proceeds and the diversity of the results attained in a span so brief.
>
> But when we have grasped this cardinal fact, we have but focussed our instruments for a study of the real problem. *How* do the adult characteristics lie latent in the germ-cells; and how do they become patent as development proceeds? This is the final question that looms in the background of every investigation of the cell. In approaching it we may well make a frank confession of ignorance; for in spite of all that the microscope has revealed, we have not penetrated the mystery, and inheritance and development still remain in their fundamental aspects as great a riddle as they were to the Greeks . . . . The real problem of development is *the orderly sequence and correlation of . . . phenomena toward a typical result.* We cannot escape the conclusion that this is the outcome of the organization of the germ-cells; but the nature of that which, for lack of a better term, we call "organization," is and doubtless long will remain almost wholly in the dark (pp. 396–397).

Yet something could be said about that organization. Since the egg is part of the parent, as Wilson emphasized, its organization must be a part of the organization of the parent. The egg has, therefore, "something" of the parents. That inherent something will be encased in a single-celled zygote and the problem becomes the mechanisms that convert the zygote to adult.

THE HYPOTHESIS OF GERMINAL LOCALIZATION

In 1874 William His (1831–1904) attempted to say something about organization. His hypothesis of germinal localization, or as it was to be called later, cytoplasmic localization, became fundamental in analytical embryology. He worked mainly with chick embryos and his problem was the eternal one—if the body of the chick is not preformed in the germ, what is? He suggested that, if the parts were not preformed, whatever is responsible for them is present at the beginning of development.

> It is clear, on the one hand, that every point in the embryonic region of the blastoderm must represent a later organ or part of an organ, and, on the other hand, that every organ developed from the blastoderm has its preformed primordium in a definitely located region of the flat germ-disc . . . . The material of the primordium is already present in the flat germ-disc, but it is not yet morphologically marked off and hence not directly recognizable. But by following the development backwards we may determine the location of every such primordium even at a period when the morphological differentiation is incomplete or before it occurs; logically, indeed, we must extend this process back to the fertilized or even the unfertilized egg. According to this principle, the germ-disc contains the primordia of the organs spread out in a flat plate, and, conversely, every point of the germ-disc reappears in a later organ; I call this *the principle of organ-forming primordial-regions.* (In E. B. Wilson's translation [1900, p. 398] of His's paper "germ" was used in two ways, one meaning embryonic, as in "germ-disc," the other referring to the substances necessary for the formation of organs. For the latter I have

substituted "primordia" for the sake of clarity.)

Today it may be hard to understand why His's hypothesis was thought important. Would not one expect that the parts of the older embryo and adult come from the substance of the zygote? What other possible source could there be? However, His was saying something more important, namely, that the organization of the egg consists of the localization of the factors, unknown but presumably material, that are responsible for the development of the parts of the embryo and adult. Thus the zygote was not to be regarded as a totally unorganized bit of protoplasm but of having some *substances*—not force or immaterial organizing principle—that were the *sine qua non* for differentiation. His was suggesting that by careful observation one could prepare a fate map of the chick embryo much as Vogt was to do a half century later for the amphibian embryo (Fig. 25).

Although His spoke of the "principle" of organ-forming germ-regions, "hypothesis" would have been a better term—he suggested, he did not prove. Nevertheless, his hypothesis was a useful way to think of the egg's organization and it suggested experimental approaches to Roux and others.

WILHELM ROUX UND *ENTWICKLUNGSMECHANIK*

Analytical embryology became a full-fledged program of experimentation in the hands of the German biologist Wilhelm Roux (1850–1924). He was a gifted, vigorous, outspoken, and dedicated scientist who was prominent—even in the Germany of his famous teacher, Ernst Haeckel. Roux's main hypotheses were to require much modification and many of his experiments proved to be defective but with brilliance and perseverance he raised the questions that brought experimental embryology into full flower. He initiated and for years was the editor of the first important journal devoted to analytical embryology—*Wilhelm Roux' Archiv für Entwicklungsmechanik,* which began in 1894–1895 and continues to this day.

Together with his compatriot, August Weismann, he developed the first important hypothesis of differentiation from which deductions could be made and then tested by observation and experiment. The Roux-Weismann hypothesis (usually called "theory") was based mainly on the observations, experiments, and interpretations of Roux plus some theoretical elaboration by Weismann.

Roux's key paper for the discussion that follows was published in 1888 and can be found, in translation, in Willier and Oppenheimer (1964). The following quotations are from that source. Roux posed the problem as follows:

> The following investigation represents an effort to solve the problem of self-differentiation—to determine whether, and if so how far, the fertilized egg is able to develop independently as a whole and in its individual parts. Or whether, on the contrary, normal development can take place only through direct formative influences of the environment on the fertilized egg or through the differentiating interactions of the parts of the egg separated from one another by cleavage (p. 4).

Roux was posing fundamental questions. The first one, whether or not the development of an egg requires specific stimuli from the environment, may seem strange to us today. It was not strange in the 1880s. Botanists had been describing the many diverse effects of the environment on the growth and differentiation of plants. Light had a pronounced effect on the production of chlorophyll, the rate of growth, the pattern of growth, leaf retention or loss, and seemingly just about everything plants did. Gravity, temperature, wind, moisture, and soil chemistry all had great effects on plant growth and development. Roux sought to determine if frog embryos were similarly affected by these environmental factors by rotating the embryos constantly so that gravity, light, heat, and magnetic forces would not be able to exert an effect from a constant direction. The embryos developed perfectly normally.

We can conclude from this that the typical structures of the developing egg and

> embryo do not need any formative influence by such external agencies for their formation, and that in this sense the morphological development of the fertilized egg may be considered as self-differentiation (p. 4).

Having answered his question for the embryo as a whole, Roux sought to ask the same question for its parts. The very fact that he was able to ask such a question at all depended not only on his work but also on the work of those who had preceded him or who were his contemporaries. We must never forget this most important aspect of scientific work. The questions that can be asked at any time relate to the state of the field, which means that others have prepared the groundwork for the scientist's research. For example, in the 1880s there were exciting new discoveries in cell biology, especially about chromosomes. It was becoming ever more important to solve the problem of inheritance. A huge amount of information was available about development and His had postulated that differentiation depended on the presence of determinants for the structures of the embryo.

All of this information is general and could not suggest to Roux what he should do the next morning when he went to his laboratory. However some very specific facts that he had learned suggested the possibility of a truly impressive experiment that, if successful, would throw great light on the age old problem of the causes of differentiation.

Roux reported that he had discovered some fascinating rules involving the early development of frog embryos. The first of these was that the plane of first cleavage coincides with the median plane of neurulae and later embryos. There was even the possibility of this being a rule of broad applicability because others had found the same thing to hold true for such different embryos as those of bony fish and ascidians. Newport had discovered this long before and Roux notes,

> It is worth mentioning that observations pertinent to this matter had already been recorded in the posthumous papers of G. Newport, published in 1854. These aroused no notice at the time and were not discovered again until later (p. 6).

This neglect of Newport's discovery cannot be explained away, as in the case of Mendel's work, as the consequence of his results being published in an obscure journal. *The Philosophical Proceedings of the Royal Society of London* was the most prestigious scientific journal in the English language. No notice was taken of Newport's discovery in 1854 because no one had the remotest idea of how to profit by it. The field was "not ready."

Roux confirmed, for the most part, Newport's other discovery of the relation of the point of sperm entrance to the plane of first cleavage and the future polarity of the embryo. But Roux found another relationship: shortly after fertilization a broad crescent in the lower part of the animal hemisphere, opposite the point of sperm entry, loses some of its dark pigment and becomes the gray crescent. The gray crescent persists at most for a few cleavages. By preventing the embryos from changing position, Roux found that the dorsal lip of the blastopore appears where the gray crescent had been.

There seemed, therefore, to be these relations. 1. The sperm enters the ovum. 2. The gray crescent forms 180° from the sperm's entrance point. 3. The plane of first cleavage is in the meridian of the entrance point of the sperm and the animal pole. 4. The plane of first cleavage bisects the gray crescent. 5. The dorsal lip forms where the gray crescent had been. 6. The anterior–posterior axis of the embryo forms in relation to the dorsal lip. Later, when the neural folds form, the blastopore will be at their posterior end.

Thus, as Newport had observed and Roux confirmed, the plane of first cleavage divides the embryo into a right and left half. Roux saw the possibility of testing His's hypothesis.

TESTING THE HYPOTHESIS OF HIS

If we assume that the hypothesis of His—that the primordia are absolutely necessary for the formation of the parts of the embryo—is true, then this deduction follows logically:

> *If some of the primordia can be destroyed, and the embryo still be able to develop to some extent, the structures normally determined by those primordia must be absent.*

Since the primordia were hypothetical structures it was impossible to identify and then manipulate them. Roux sought to achieve that end, however, in an indirect way. This involved a subsidiary hypothesis and this deduction:

> *If the plane of first cleavage divides the embryo into a right and left half, each half must contain the primordia for that specific half. Therefore, the destruction of one blastomere would also destroy the primordia for half of the body.*

After trying various methods Roux destroyed one cell of the two-cell stage with a hot needle.

> I heated the needle by holding it against a brass sphere for a heat supply, heating the sphere as necessary. In this case only a single puncture was made, but the needle was ordinarily left in the egg until an obvious light brown discoloration of the egg substance appeared in its vicinity . . . I now had better results; they were as follows. In about 20% of the operated eggs only the undamaged cell survived the operation, while the majority were completely destroyed and a very few, where the needle had possibly already become too cold, developed normally. I thus developed and preserved over a hundred eggs with one of their halves destroyed, and, of these, 80 were sectioned completely (p. 9).

And the experimental analysis of development was underway. In the 20 percent where the untreated cell survived, various results could be expected,

> For example, abnormal processes might intervene which would lead to bizarre structures. Or the single half of the egg, which, after all, according to many authors, is a complete cell with a nucleus completely equivalent in quality to the first segmentation nucleus, might develop into a correspondingly small individual. These authors see in the mechanism of indirect nuclear segmentation [*i.e.*, mitosis], on my authority as it were, only a contrivance for qualitative halving. I have repeatedly and clearly opposed this opinion. But instead of the possible surprises as postulated above an even more amazing thing happened; the one cell developed in many cases into a half-embryo generally normal in structure, with small variations occurring only in the region of the immediate neighborhood of the treated half of the egg (p. 12).

Figure 35 shows some of the results. In embryo *a* the left blastomere had been killed but the right blastomere lived and formed a half blastula. In embryo *b* the right blastomere had been killed, was later sluffed off, and the living side rounded up and produced an embryo with a single neural fold and with the mesodermal layer extending from the notochord around the left side of the embryo only. There is what may be described as half an archenteron, though it is hard to recognize half a hole. What was one to conclude?

> In general we can infer from these results that each of the first two blastomeres is able to develop independently of the other and therefore does develop independently under normal circumstances . . . . All this provides a new confirmation of the insight we had already achieved earlier that developmental processes may not be considered a result of the interaction of all parts, or indeed even of all the nuclear parts of the egg. We have, instead of such differentiating interactions, the self-differentiation of the first blastomeres and of the complex of their derivatives into a definite part of the embryo . . . . The development of the frog gastrula and of the embryo initially produced from it is, from the second cleavage on, a mosaic of at least four vertical pieces developing independently (pp. 25–28).

Figure 36 is a schematic representation of

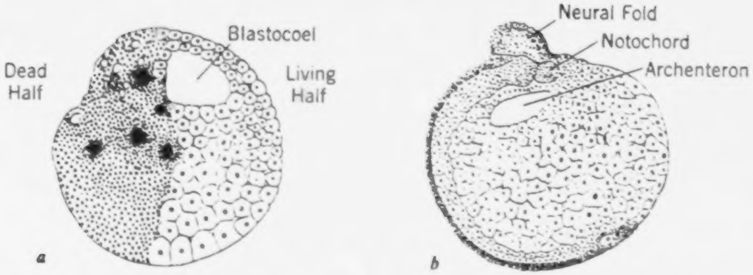

FIG. 35. Half-embryos obtained after killing one blastomere of the two-cell stage of a frog embryo. In A the dead half remains. In B it has been sluffed off. (Roux, 1888, p. 113.)

Roux's interpretations after it came to be assumed that the determinants were associated with the nucleus. It shows the segregation of the determinants that produces "a mosaic of at least four vertical pieces developing independently."

These results can be taken as a dramatic and convincing test of our original two deductions and so Roux's hypothesis for the localization of determinants is made more probable—as is, of course, His's hypothesis that there are primordia, or determinants, for differentiation.

DIFFICULTIES WITH THE HYPOTHESIS

It is hard to overemphasize the importance of Roux's hypothesis for the rapidly developing field of experimental embryology. However, important ideas in science must be tested in a variety of ways and by many different scientists before they can be accepted. Such requirements help to eliminate faulty hypotheses and faulty experiments. Roux's ideas were center stage for at least a decade but eventually they had to be drastically altered because

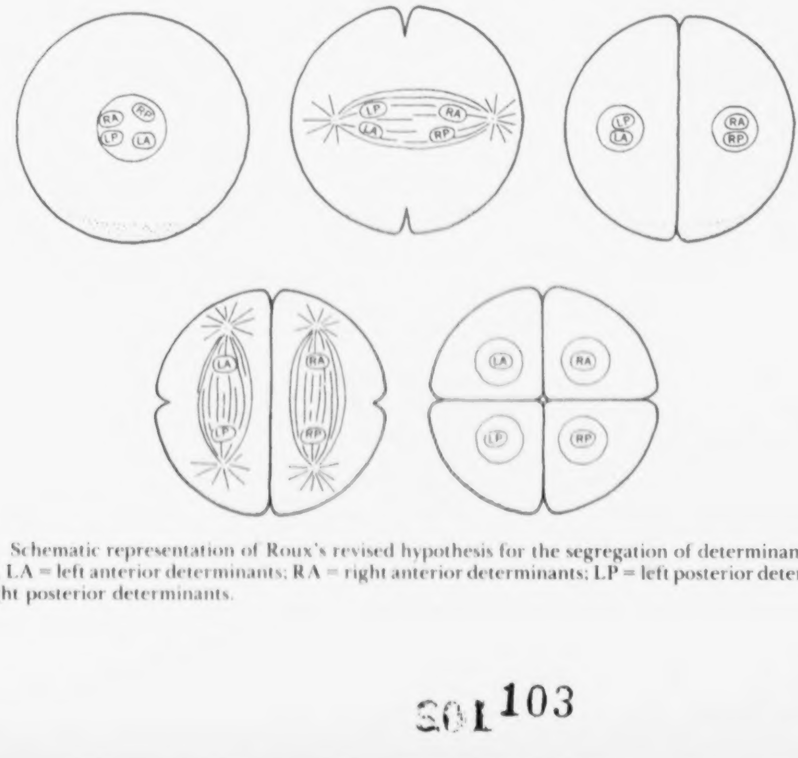

FIG. 36. Schematic representation of Roux's revised hypothesis for the segregation of determinants during cleavage. LA = left anterior determinants; RA = right anterior determinants; LP = left posterior determinants; RP = right posterior determinants.

of what he and others discovered. These later developments will be summarized now, out of place so to speak, and then we will return to the main line of analysis pretending to be ignorant of what is to come.

Roux's hypothesis appeared to be fully confirmed by the development of the half-embryos up to the neurula stages. Some of the embryos, however, were kept and what was surely a most discouraging phenomenon was observed: the half-embryo gradually formed a whole embryo. Roux called this "postgeneration."

The simplest interpretation of postgeneration would be that there had been no destruction of the determinants for one side. It had been assumed that each cell of the two-cell stage had the determinants *only* for that half. Certainly development to the neurula stage seemed to indicate that the determinants for the operated side had been destroyed. If so, they could not have "come to life" and produced a whole embryo. Yet they must have been preserved in some way since postgeneration would have been impossible without them.

Roux developed a subsidiary hypothesis to account for postgeneration but the fact that he had to do so greatly weakened his original hypothesis. He held, in effect, that there were two sorts of determinants. The main sort consisted of the determinants that were divided *qualitatively* during cell division and specified the organs and parts of the embryo. In addition, another sort of determinant was held in reserve. It was divided *quantitatively* and kept intact the complete set of determinants. Later in development, if a part were lost, this reserve set made it possible for there to be a complete regeneration.

This *deus ex machina* solution was without much merit. It was proposed to save the original hypothesis and it seems impossible that experiments could be devised to test it.

Roux himself reported some experiments that made the original hypothesis questionable when he found that embryos could be obtained, without the need for any postgeneration, from single cells of the two-cell stage. (He called these "hemioholoplasten" embryos—a term that, seemingly, was not widely accepted and used.)

Eventually the data made it less and less likely that Roux's hypothesis was correct and others began to suggest another, namely:

> *There is no segregation of determinants in the early cleavage stages.*

If this alternative hypothesis is true, it would still be necessary to explain the results Roux obtained in the embryos developing to the neurula stage—results that were confirmed when others repeated Roux's experiments. One possibility is that these half-embryos might be the result of the presence of the dead cell affecting the development of the living cell. If so, the following deduction can be made:

> *If one cell of the two-cell stage is removed rather than destroyed, the remaining cell should produce a complete embryo.*

Various ways of performing this experiment were tried and the clearest results were obtained by McClendon (1910). He found it possible to remove one blastomere at the two-cell stage by sucking one out with a tiny pipette. The single remaining cell developed and produced a complete, though small, embryo. Morgan (1897, pp. 111–122) reports on the early attempts to confirm Roux's claims and provides a later (1927, ch. 18) account of the clearing up of the problem.

For the time being we will ignore these final clarifications since they were unknown and Roux's experiments and conclusions played an important role in the analysis of development.

SCRAMBLING THE EGGS

In 1884 Pflüger found that, if uncleaved frog eggs were compressed between two plates of glass, the planes of cleavage could be modified. This technique allowed the experimenter to study the role of cleavage in later development. It proved to be so valuable that it was employed by many workers who used it on a wide variety of eggs.

Figure 37 shows one such experiment in which an uncleaved frog egg, in side view,

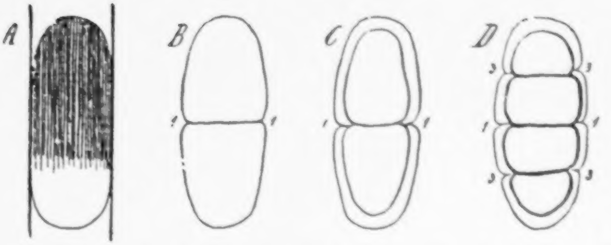

FIG. 37. The effects of pressure on the planes of cleavage. The animal hemisphere is shaded in A. Orientation is the same in B–D. The numbers 1 and 3 indicate the planes of first and third cleavage. The second cleavage plane is vertical (C) but is not numbered. (From Jenkinson, 1909, p. 36.)

is compressed by two plates that are parallel to the animal pole–vegetal pole axis. The animal hemisphere is shaded. "B" shows the plane of first cleavage extending horizontally from one glass plate to the other. In an uncompressed embryo this cleavage would have been vertical. "C" shows second cleavage, which is vertical. In "D" the third cleavage again is horizontal. The result is a very strange eight-cell stage.

It may seem surprising but there is a simple explanation for these abnormal cleavage planes: the mitotic spindle takes a position with its long axis parallel to the two pressure plates. In "B" and "D" the spindles would have been parallel and vertical and in "C" parallel and horizontal.

This technique allowed one to control the positions of the early cleavage planes and so scramble the relative positions of the blastomeres. This afforded a test of Roux's hypothesis for the localization of the primordia of the embryo's structures and their qualitative separation by cell division (Fig. 36). Recall that Roux maintained that the frog embryo is "from the second cleavage on a mosaic of at least four vertical pieces developing independently." One could further test his hypothesis by the following deduction:

> *If regional determination is a consequence of the separation by the process of cleavage of the determinants, or primordia, for future differentiation, any modification of the planes of cleavage should be followed by specific modifications of development.*

However, when these pressure experiments were performed normal embryos developed.

These experiments appeared to be fairly conclusive in showing that Roux's hypothesis was probably wrong. A precise pattern of cleavage, dividing those hypothetical determinants, was not a requirement for normal development. There was, however, another possibility. It could be maintained that the determinants are localized in specific portions of the uncleaved egg and that they are not segregated by cell division. They would eventually be in the proper place no matter what the earlier planes of cleavage had been. The role of cleavage would then be solely to divide the original egg into many smaller cells, and cleavage would not be a primary mechanism for differentiation—the embryo would still be a mosaic of independently developing parts.

FURTHER TESTS OF THE HYPOTHESIS OF MOSAIC DEVELOPMENT

Despite such results, and the difficulty in explaining postgeneration, Roux's dramatic experiments and seemingly reasonable hypothesis were having a great influence. If his conclusions could be established as true beyond all reasonable doubt, embryologists would be well on the way to solving the problem of differentiation. It was hoped, of course, that a general explanation was at hand and not one that applied only to a single species of European frog. The hypothesis of mosaic development remained the prime concept of the last quarter of the 19th century and many investigators, working on many sorts of organisms, sought to test it further. The resulting studies fell into two main classes: Roux was right; Roux was wrong. There were dramatic verifications and rejections. We will start with one of each.

A HARMONIOUS EQUIPOTENTIAL SYSTEM

Four years after Roux's seminal paper, Hans Driesch (1892) sought to confirm or deny Roux's hypothesis using a European sea urchin, *Echinus microtuberculatus.* He, too, started with the hypothesis of His's organ-forming primordial areas and, as had Roux, sought to separate the cells of the two-cell stage.

Driesch's methods were considerably different. Instead of killing one of the cells, he put about 100 embryos in a small tube with a little sea water. Then he shook the tube violently for about five minutes or more. Some of the two-cell embryos were found to have broken through their membranes and the blastomeres had separated. From many experiments he was able to obtain for study about 50 isolated cells from the two-cell stage.

How would they develop? The first check could be made of the cleavage stages. In normal embryos the planes of the first two cleavages are vertical and the third horizontal, as in the frog. The result is eight cells of almost equal size. The fourth cleavage, however, is very different. The four vegetal cells divide very unequally to give four large macromeres and four very small micromeres. The four animal hemisphere cells divide about equally.

Would the isolated blastomeres of the two-cell stage produce micromeres and, if so, at which division? If they behaved as whole normal embryos, they would form micromeres at the fourth division. If they behaved as though they were still part of a whole embryo, the micromeres would be formed at their third division (the isolated cells would, of course, have already gone through the first division and then been shaken apart). Driesch found that the first two cleavages were equal, giving four cells looking just like a half of a normal embryo. The third cleavage of the isolated cells produces micromeres and the result was an embryo that was identical with half of a normal 16-cell embryo.

So far, the isolated cells were behaving just as His and Roux would have predicted. In fact they continued to exhibit mosaic development, forming a half blastula, that is, one that resembled a cup in being open on one side.

That was the evening of the first day and, having observed the experimental embryos all day, Driesch went to bed. What would the morrow bring when he knew that normal embryos would gastrulate and develop into pluteus larvae? By now he was down to 15 experimental embryos, some of the others having been preserved for study or died. He wondered if those remaining would be half gastrulae and half pluteus larvae.

Three had formed *fully normal* pluteus larvae except for their small size. Apparently Driesch was astonished but not overjoyed with this discovery. It seemed to him "almost a step backward along a path considered well established."

How could he account for these results? Driesch suggested that, after all, frogs are not sea urchins. Since this answer did not seem adequate, he thought that maybe the difference was that Roux had not really isolated blastomeres at the two-cell stage. He had killed one cell, but it remained in contact with the one living one. Possibly the dead one was having an inhibitory effect.

It seemed possible to Driesch that the two sorts of embryos would have behaved in the same manner if the blastomeres in both had been fully isolated. (His guess was correct for, as we have already seen, years later McClendon and others were to succeed in removing one cell of the two-cell stage of frog embryos and find that normal larvae would result.)

These results were extremely difficult to deal with. When the cells of the two-cell stage remained together, each would produce half of an older embryo. Yet when these same kinds of cells were separated from one another, after developing as half-embryos through the blastula stage, they regulated and formed entirely normal pluteus larvae. One had to assume that there is some overall control exerted by the whole embyro over its constituent parts, that is, the embryo is not a complete mosaic of self-differentiating parts.

Thus, there must be some harmonious control by the entire embryo of its equipotential parts—a hypothesis that led Driesch to regard the developing sea urchin embryo as a "harmonious equipotential system." But this was not to be true of some other invertebrate embryos.

CTENOPHORES

The ctenophores are beautiful, medusa-like, marine invertebrates. Their bodies are of glass-like transparency. They move slowly through the ocean water propelled by eight rows of comb plates, which are a series of paddle-like structures that, in fact, serve as paddles.

Several investigators had isolated the blastomeres of ctenophore eggs, among them Driesch and Morgan (1895). They used *Beroe ovata* in which the first three cleavages are vertical, producing an eight-cell stage with the blastomeres almost in a flat plane. Morgan (1897, pp. 129–130) summarized their results:

> When the first two blastomeres are separated from each other by a sharp needle or cut apart by a pair of small scissors, each continues to cleave as a half, *i.e.* as though it were still in contact with its fellow-blastomere. When the organs appear in the larva, only half the full number of rows of swimming-paddles appear. Each row, however, has its full complement of paddles. The invagination of ectoderm to form the "stomach" is very excentric in the half-larva, but forms a *closed* tube running from the mouth-opening to the excentric sense-plate. In several respects, therefore, the larvae were distinctly half-larvae. But in other respects they were more than half-larvae. The endodermal cells of the normal larva arrange themselves into four hollow pouches, and the "stomach" invagination passes in the central line of the four pouches. In the half-larva, on the contrary, the endodermal mass forms more than two pouches (*i.e.* more than half the number in the whole larva). Two distinct pouches are present and in addition, generally, a third smaller pouch is formed . . . .
>
> The isolated one-fourth blastomere [that is, one blastomere from the four-cell stage] segments also as a part of a whole, and develops in some cases into a one-fourth larva, having only *two rows* of paddles (*i.e.* one-fourth the normal number), but with *two* endodermal pouches . . . . The three-fourth embryos [three cells of the four-cell stage] develop six rows of paddles and *four* endodermal pouches . . . .
>
> The results show, however, beyond question, that, even when isolated from its fellow, the one-half blastomere may give rise to a larva that is in many respects only one-half of the normal larva.

The fact that the isolated blastomeres cleaved as though they were still part of a whole embryo, and that the number of rows of comb plates seemed to indicate strictly mosaic development, deeply impressed embryologists. Later experiments indicated (but did not make certain as it turned out; see Reverberi, 1971, pp. 85–103) that each isolated cell from the eight-cell stage would produce a larva with one row of comb plates. That was about as mosaic as one could get.

REGULATIVE AND MOSAIC DEVELOPMENT

Clearly sea urchin and ctenophore embryos were different. It appeared that there were two fundamentally different patterns of development—mosaic and regulative. The first was a pattern of independently developing parts and the latter a pattern of parts that could regulate and form more than they were normally destined to do. The ctenophore embryo was taken as an example of mosaic development and the echinoderm egg as an example of regulative development.

Regulative development was a disturbing notion. What could be the controlling mechanism that restrained the individual cells of the two-cell sea urchin embryo and molded them into parts of a single organism but released those same cells when isolated and allowed each to form an entire organism? It had all seemed so clear and intellectually satisfying if development were, as Roux suggested, fixed from the onset. It had been equally satisfying, long before, when it was accepted that the embryo was preformed in the ovum (or sperm) and equally disturbing when finally it was shown convincingly that development is epigenetic.

The concepts of preformation and mosaic development avoided the central problem of development—how can novelty arise? The concepts of epigenesis and regulative development must come to grips with that central problem.

Driesch puzzled about the implications of his discoveries, that backward step as he saw it, and he eventually abandoned experimental science and devoted full time to philosophy.

DEVELOPMENT IN AMERICA

Experimental embryology began as a European, and mainly German, science. Newport had been forgotten and the field was dominated by Wilhelm His, Eduard Pflüger, Wilhelm Roux, Hans Driesch, Gustav Born, Oscar and Richard Hertwig, Alexander Kowalewski, Curt Herbst, and Edouard van Beneden. Most of these individuals were associated with universities, and many trained graduate students.

Universities are traditionally the nurseries of science, and in the last quarter of the 19th century a few American universities began to develop programs in biology. Johns Hopkins was the most notable example. It became possible to be trained as a professional embryologist in the United States. Beginning with Charles Otis Whitman, who however had studied with Rudolf Leuckart in Leipzig, a vigorous school of American embryologists developed that by the 1880s was engaged in outstanding research. The group included Edward Beecher Wilson (1856–1939), Thomas Hunt Morgan (1866–1945), Edwin Grant Conklin (1863–1952), and Ross Granville Harrison (1870–1959). All had studied with William Keith Brooks (1848–1908) and Henry Newell Martin (1848–1896) at Johns Hopkins. These four students did much to make embryology and genetics more exact sciences.

The origin of the American school of embryologists was not an example of mosaic development, with Europe and America showing self-differentiation. The fledgling American school had the benefit of the vast literature of descriptive embryology, all European, and its members visited the European laboratories and the marine station at Naples where they met the outstanding European embryologists. Thus the Americans became part of international experimental embryology.

AMPHIOXUS

In the summer of 1892 E. B. Wilson (1893) worked at the Statzione Zoologica, a marine biological laboratory at Naples, and repeated Driesch's experiments, using the cleavage stages of amphioxus (=*Branchiostoma*). His basic technique was the same—that of shaking the blastomeres apart. This is what he found:

> An isolated ½ blastomere [that is, one cell of the two-cell stage] undergoes a cleavage identical with, or approximating to, that of a normal embryo. It produces a normally-formed blastula [in contrast with the sea urchin] and gastrula of half the normal size, and finally may give rise to a half-sized dwarf larva exactly agreeing, except in size, with the normal larva up to the period when the first gill-slit is formed . . . .
>
> An isolated ¼ blastomere may undergo a cleavage nearly or quite identical with that of a normal ovum, but often varies more or less widely from it. It may give rise to a ¼ blastula and ¼ gastrula, differing from the normal only in size. The [larval] stage, with a notochord, is rarely attained and no normally constituted ones were observed . . . .
>
> The ⅛ blastomeres are of two sizes (micromeres and macromeres) which, as far as could be determined, do not differ essentially in mode of development. The isolated blastomere segments in a form approaching that of a complete ovum but *never identical with it.* In rare cases a ⅛ blastula is formed . . . but the gastrula stage is never attained (pp. 587–589).

Wilson then provides an analysis of the Roux-Weismann hypothesis, especially as it applies to postgeneration and to regeneration in general. These two phenomena were very difficult to explain on the basis of that hypothesis. Wilson suggested that the hypothesis was based on two main assumptions—both false.

> The first assumption relates to the causes of histological differentiation. It is assumed [by Roux] that in normal development differentiation is primarily determined by the nature of cell-division, karyokinesis [= mitosis] being conceived as qualitative in character in such wise that cells of different prospective value receive correspondingly different forms of idioplasm [the hereditary material, whatever it might be] at the moment of their separation. Every cell, therefore, has an independent power of self-determination inherent in the structure of its idioplasm, and this in turn owes its character to the nature of the mitosis by which the cell-nucleus arose. The entire ontogeny is, therefore, compared by Roux to a mosaic work; it is essentially a whole arising from a number of independent self-determining parts . . . .
>
> The second of the Roux-Weismann assumptions is logically necessitated by the first in view of the phenomena of regeneration. Obviously these phenomena are inexplicable under a theory of strictly qualitative division. Both Weismann and Roux, therefore, assume that during cell-division each cell may receive, in addition to its specific form of idioplasm, a portion of unmodified idioplasm afforded by purely quantitative division. This unmodified idioplasm . . . remains latent in normal development . . . . Injury to the ovum . . . acts as a stimulus to the latent idioplasm, which thereupon becomes active, and causes a repetition of the original development.
>
> Considered as a purely formal explanation, the Roux-Weismann hypothesis is perfectly logical and complete. Its weakness lies in its highly artificial character; for both of its two fundamental postulates—viz: qualitative nuclear division, and accessory latent idioplasm—are purely imaginary. They are complicated assumptions in regard to phenomena of which we are really quite ignorant, and they lie at present beyond the reach not only of verification, but also of disproof (pp. 605–606).

It is not sufficient to demolish one explanatory hypothesis without providing another so Wilson provided one. He adopted a line of reasoning that he modified over the years and that became, I believe, a paradigm of differentiation that remains central to this day. He proposed to give "a simpler and more natural interpretation of the facts" that was similar to the views already adopted by Oscar Hertwig and Driesch. He assumed that mitosis

> is not qualitative, but purely quantitative; that at every cell-division the daughter cells, whatever their prospective character, receive exactly equal kinds, as well as amounts, of nuclear material . . . [differentiation is] a result of *physiological* changes in the idioplasm, *subsequent to cell-division,* such that certain of the idioblastic units [equivalent to today's genes] remain latent, while others become active and determine the specific form and activities of the cell. Finally, the physiological specialization of the idioplasm is brought about by the interaction of the cell with its fellows in the cell-complex . . . [many experiments have shown that the] embryo develops as a whole, as a unit, and demonstrate the truth of the principle urged by Whitman, Hertwig and others, that "the organism, as a whole, controls the formative processes going on in each part" (pp. 606–607).

Thus the results of Driesch on isolated sea urchin blastomeres and of Wilson on amphioxus found a ready explanation. If all cells receive the same hereditary materials and if embryos act as wholes, a single cell from the two-cell stage should behave as a whole.

But this does not hold true as development progresses. Wilson's experiments showed that for blastomeres isolated up to the eight-cell stage, "their power of development progressively diminishes as the cleavage advances" (p. 608). This was thought to be a consequence of the following:

> As the ontogeny advances the idioplasm of the cells undergoes gradual and progressive *physiological* modification (brought about by the interaction of the various parts of the embryo), without, however losing any of its elements. The isolation of a blastomere restores it in a measure to the condition of the original ovum and the idioplasm, therefore, tends to return to the condition of the original germ-plasm and thus to cause a repetition of the development from the beginning.

But as development continues the idioplasm becomes progressively modified.

> By the 8-celled stage [in amphioxus] it is incapable of returning to the original state, and the normal type of cleavage is no longer repeated . . . . The specialization of the idioplasm, like that of the cell as a whole, appears to be a cumulative process that results in a more and more fixed mode of action . . . . The independent, self-determining power of the cell, therefore, steadily increases as the cleavage advances. In other words: *the ontogeny assumes more and more of the the character of a mosaic-work as it goes forward. In the earlier stages the morphological value of a cell may be determined by its location. In later stages this is less strictly true and in the end the cell may become more or less completely independent of its location, its substance having become finally and permanently changed* (pp. 606–610).

Wilson pushes the analysis back to the beginning of development by suggesting that we regard

> ontogeny as a connected series of interactions between the blastomeres in which each step conditions that which succeeds. The character of the whole series depends on the first step, and this in turn upon the constitution of the original ovum . . . . The entire series of events is primarily determined by the organization of the undivided ovum that forms its first term, and, as such, conditions every succeeding term (pp. 613–614).

CELL LINEAGE

Few observations speak so forcefully for the importance of the organization of the ovum as those on cell lineage, which were one of the main contributions of the American school in the 1890s and early 1900s. Cell lineage is the description of the history of each embryonic cell. Beginning with the uncleaved egg, the products of every division are traced until the rudiments of the embryonic organs have become distinct.

Suppose that a hypothesis we are testing requires that we know the origin of every cell in an early embryo, that is, the ancestors of each cell back to the uncleaved zygote. Let's take the frog embryo as an example to show what might be done and some of the problems that would arise.

We would begin with the uncleaved egg, as in Figure 17. First cleavage divides the embryo into two identical cells yet, because of the presence of a gray crescent, we could distinguish them. Thus, if we view the embryo from the gray crescent side, we could call one blastomere "right" and the other "left." (Had there been no gray crescent, there would be no way to differentiate the first two cells.) In the case of the frog egg we would know, thanks to Newport and Roux, that if we view the egg from the gray crescent side, the right blastomere will form the right half of the embryo and the left blastomere the left half of the embryo.

The second cleavage divides the embryo into an anterior-right blastomere, posterior-right blastomere, posterior-left blastomere and anterior-left blastomere (Fig. 36). The third cleavage is horizontal and forms an upper-right-anterior blastomere, lower-right-anterior blastomere, upper-right-posterior-blastomere, lower-right-posterior blastomere, etc. And by this eight-cell stage it is clear that a better system of identifying embryonic cells is essential.

Moreover by this time the gray crescent is difficult to recognize and, unless the cells are vitally stained, we could no longer work out their lineages. Frog embryos are unsuitable for another reason. They are opaque, which makes it impossible to observe cells in the interior.

Only if individual cells can be identified throughout early development is it possible to trace their lineage. Such embryonic cells must differ in some way from one another, either in size, coloration, or position. As embryologists coursed up and down the animal kingdom looking for suitable embryos to study, they found many, especially those of marine invertebrates, with distinctive patterns of coloration and cleavage, and with different sizes of blastomeres. Some embryos were even transparent, allowing one to observe cells of the interior.

Therefore nature was providing naturally stained eggs that could serve the same purpose as Vogt's vitally stained embryos. The patterning of pigmentation of the eggs was found not to be a random affair but part of a basic organization. The planes of cleavage were constant in relation to the pigmented areas, and in many cases the differently colored regions of the egg seemed to have a fixed relation to the germ layers and to the structures that they would form.

This visible organization of certain kinds of eggs at the very beginning of development made it difficult to regard a just-fertilized ovum as an amorphous mass of protoplasm awaiting the directing influences of either idioplasm, determinants, gemmules, nuclei, chromosomes, or whatever. One could not deny organization when it was so striking and constant in what it was and did.

There was, however, an opposing view stated earlier by Pflüger. He held that the uncleaved ovum is *isotropic*, that is, there is no axial organization and all parts of the cytoplasm are equivalent. This hypothesis appealed to many investigators who were impressed by Driesch's experiments on sea urchins and some other experiments in which two eggs were fused and found to produce a single embryo.

Things were confusing! There appeared to be experimental proof for nearly every conflicting hypothesis.

CLEPSINE

One of the first of the painstaking studies of cell lineage was done by Whitman (1878) on the embryos of *Clepsine complanata* (now *Glossiphonia complanata*), a leech. The first two cleavages of *Clepsine* produce four cells of equal size, which Whitman called *a*, *b*, *c*, and *x*. At the next division these divide to give four very small cells and four large ones. The four small cells are the progenitors of the ectoderm. The following cleavages become irregular. The cell derivatives of *x* were able to be followed and were found to give rise to the mesoderm and the nervous system. In fact, Whitman found that entire organ systems could be traced back to their origin in pairs of cells, called teloblasts. One pair gave rise to the mesoderm bands, another pair to ventral nerve cord, another to the trunk nephridia, and so on.

Although Whitman was only doing descriptive embryology, in this case carried out in great detail, he also related his observations to the explanatory hypotheses of the time:

> In the fecundated egg slumbers potentially the future embryo. While we cannot say that the embryo is predelineated, we can say that it is predetermined. The "Histogenetic sundering" of embryonic elements begins with the cleavage, and every step in the process bears a definite and invariable relation to antecedent and subsequent steps . . . . It is, therefore, not surprising to find certain important histological differentiations and fundamental structural relations anticipated in the early phases of cleavage, and foreshadowed even before cleavage begins.
>
> The egg is, in a certain sense, a quarry out of which, without waste, a complicated structure is to be built up; but more than this, in so far as it is the architect of its own destiny (pp. 263–264).

Whitman expressed a point of view that His had proposed in 1874 and Roux held a few years later: the parts of the future embryo existed as primordia from the very beginning and the course of development is determined, not regulative.

NEREIS

In 1892 E. B. Wilson published a magnificent study of the cell lineage of the marine polychaete worms, *Nereis limbata* and *Nereis megalops*. These he collected in the Eel Pond behind the Marine Biological Laboratory at Wood's Holl (as it was called then; it later became Woods Hole). He referred to the "epoch-making researches" of his friend Whitman, who had become the director of the MBL, noting:

> That an entire system of organs, such as the ventral nerve-cord, or trunk-nephridia could be traced back to a single blastomere was a fact so extraordinary that many morphologists, Balfour among them, at first refused to credit Whitman's statements . . . . Whitman's researches showed that the material for complicated adult organs might be so condensed and accelerated in development as to be set apart by a single stroke, as it were, in the early stages of cleavage, long before the establishment of the gastrula (p. 368).

Wilson also was working as a descriptive embryologist, yet his findings were to be of great importance in analytical embryology. He studied *Nereis* in the hopes of learning more about the homologies of the germ layers.

First cleavage cuts across what will become the future longitudinal axis of the embryo, dividing the egg into a small anterior cell, called *AB*, and a large posterior cell, called *CD* (Fig. 38A). The second cleavage coincides with the median plane of the future body and it produces four large macromeres: *AB* dividing into *A* and *B* and *CD* into *C* and *D* (Fig. 38B, C). Third cleavage (Fig. 38D) is horizontal and unequal. Each large macromere gives off a small micromere. This first quartet of micromeres Wilson designated as a^1, b^1, c^1, and d^1.

Each micromere does not come off directly above a macromere. Instead each comes off in a slightly clockwise direction. This pattern is known as "spiral cleavage."

It might be suspected that this clockwise movement of the micromeres is merely their sliding into the grooves between the almost spherical macromeres but this is not the case. Before the third cleavage began the spindle in each macromere was slanted in a clockwise direction as much as 45°. Spiral cleavage is a reflection of the embryo's basic organization, not a device for convenient packaging of the blastomeres.

The fourth division is also unequal and horizontal. This time the spindles of the macromeres slant in the opposite direction and a second quartet of micromeres comes off in a counterclockwise direction. At the same time the first quartet of micromeres divides.

At the fifth division, the third quartet of micromeres comes off the macromeres in a clockwise direction.

These first three quartets of micromeres form the entire ectoderm.

At the next division, which is no longer synchronous throughout the embryo, the *D* macromere divides into a large cell, still called *D*, and a smaller cell, d^4 (the bottom embryo in Fig. 38; this has been simplified by omitting the divisions of the micromeres). That d^4 was to become famous, because localized in that small cell was the entire material that would form mesodermal structures.

> The formation of the second somatoblast [= d^4] ends the spiral period of development, and it is a very significant fact that the close of this period marks also the complete differentiation, not only of the germ-layers, but also of many of the protoblasts from which the adult organs arise. The segregation of the embryonic material is in fact so nearly completed, that this last spiral stage may be taken as a new point of departure. The embryo now consists of thirty-eight blastomeres (pp. 392–393).

with their fates as summarized in Figure 39.

Wilson used terms ending in "blast" to designate "a blastomere of the segmenting

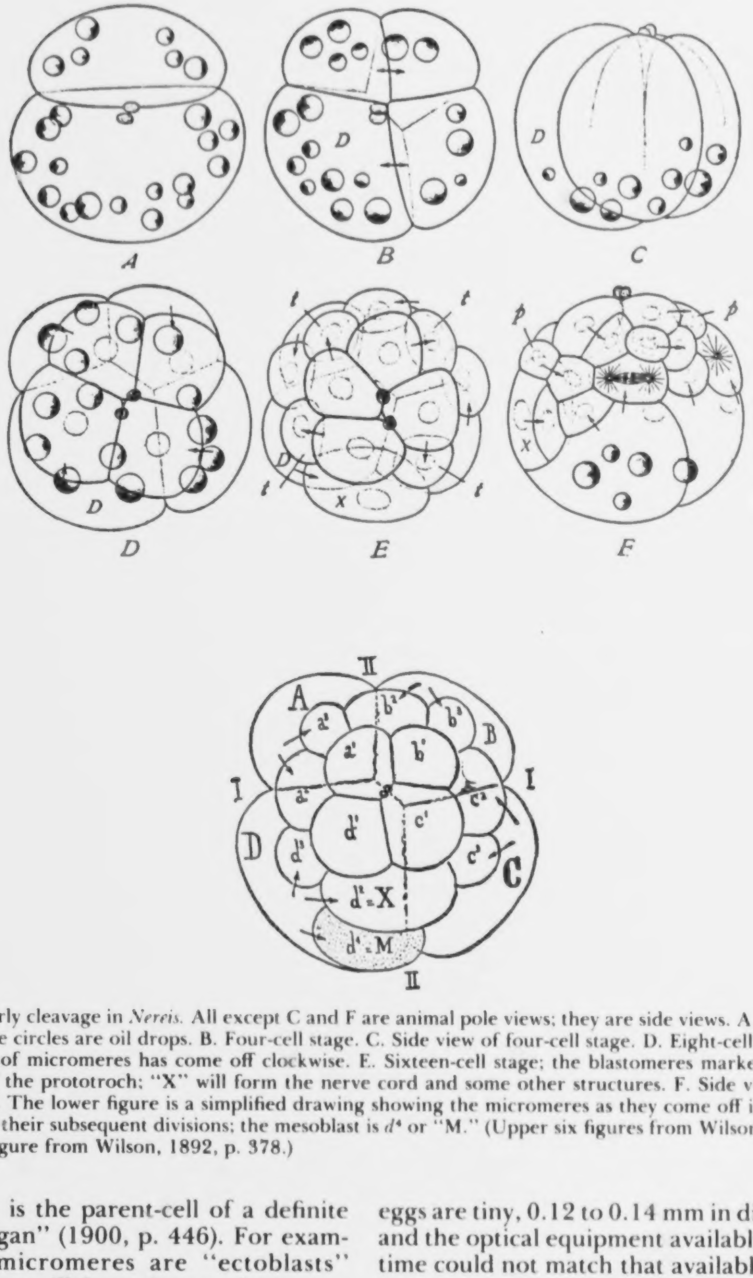

FIG. 38. Early cleavage in *Nereis*. All except C and F are animal pole views; they are side views. A. The two-cell stage; the circles are oil drops. B. Four-cell stage. C. Side view of four-cell stage. D. Eight-cell stage; the first quartet of micromeres has come off clockwise. E. Sixteen-cell stage; the blastomeres marked "t" will form part of the prototroch; "X" will form the nerve cord and some other structures. F. Side view of the 29-cell stage. The lower figure is a simplified drawing showing the micromeres as they come off in quartets but omitting their subsequent divisions; the mesoblast is d^4 or "M." (Upper six figures from Wilson, 1900, p. 369; lower figure from Wilson, 1892, p. 378.)

egg which is the parent-cell of a definite part or organ" (1900, p. 446). For example, the micromeres are "ectoblasts" because they will form the ectoderm.

It is worth examining Wilson's chart (Fig. 40) that shows the complete cell lineage of *Nereis*—not so much for the details but for the amount of work that was required. The eggs are tiny, 0.12 to 0.14 mm in diameter, and the optical equipment available at that time could not match that available today. The time of breeding was most inconvenient—after dusk. The adult males and females swarm at the surface of the water where they can be netted and then placed in separate dishes. Back in the laboratory

4.	The macromeres A, B, C, D, or entomeres	= Entoblast.
4.	The first group of micromeres, a^1, b^1, c^1, d^1,	Ectomeres = Ectoblast.
8.	The products of the trochoblasts, $a^{1\cdot1\cdot1}$, $a^{1\cdot1\cdot2}$, $b^{1\cdot1\cdot1}$, $b^{1\cdot1\cdot2}$, $c^{1\cdot1\cdot1}$, $c^{1\cdot1\cdot2}$, $d^{1\cdot1\cdot1}$, $d^{1\cdot1\cdot2}$,	
4.	The four intermediate girdle-cells, $a^{1\cdot2}$, $b^{1\cdot2}$, $c^{1\cdot2}$, $d^{1\cdot2}$,	
4.	The rosette-cells, $a^{1\cdot3}$, $b^{1\cdot3}$, $c^{1\cdot3}$, $d^{1\cdot3}$,	
3.	The three smaller secondary micromeres, $a^{2\cdot1}$, $b^{2\cdot1}$, c^{21},	
3.	The stomatoblasts, $a^{2\cdot2}$, $b^{2\cdot2}$, $c^{2\cdot2}$,	
3.	The first somatoblast (X) and its progeny (x^1, x^2),	
4.	The four tertiary micromeres (a^3, b^3, c^3, d^3),	
1.	The second somatoblast or mesomere	= Mesoblast.
38		

FIG. 39. The fates of the blastomeres of *Nereis*. (Wilson, 1892, p. 393.)

when males and females were put together spawning would occur. Observations on the embryos began about 9 P.M. and continued throughout the night.

In order to trace every cell Wilson had to view the cleavage stages from all angles but manipulating the eggs was quite difficult because of their very small size. He solved this problem in an interesting manner. He placed tiny pieces of wax on a microscope slide so that they would support one edge of a cover glass. The cover glass would thus be at a slight angle. The eggs with a drop of ocean water were then drawn up into a pipette and squirted under the cover glass. The eggs arranged themselves in a single layer and they could be turned by gently moving the cover glass.

Wilson made 92 beautiful colored drawings of the embryos and these were lithographed in Frankfurt-am-Main and published in eight plates as part of his *Nereis* paper in the *Journal of Morphology*—at that time the most prominent zoological journal in the United States. This is one of the very great studies in embryology and is well worth examining not only for what is said but for its beautiful illustrations and the evidence of dedicated, careful and difficult work. (I knew Wilson when I was an undergraduate at Columbia University. And after he died most of his papers were discarded but I rescued his original drawings for the *Nereis* paper. Years later I gave them to the library of the American Philosophical Society for their archival collection in American Biology.)

Wilson was able to describe the cell lineage of *Nereis* completely. What was to be concluded?

> The cleavage of the ovum takes place with a precision and regularity which oft-repeated examination only renders more striking and wonderful [even after being up all night!] . . . . The entire ontogeny gives the impression of a strictly ordered and predetermined series of events, in which every cell-division plays a definite *role* and has a fixed relation to all that precedes and follows it (p. 377).

One might gather from these remarks that Wilson believed that development in *Nereis* is strictly of the mosaic type. The complexity and rigidity of the cleavage patterns would seem to indicate that such was the case. Did the *Nereis* embryo consist of an assemblage of determined and self-differentiating cells?

The studies of both Roux and Driesch had appeared shortly before this work of Wilson. He suggested that the difference between the development of "isolated" blastomeres of the frog and isolated blastomeres of the sea urchin was only that the regeneration of the missing half occurred much earlier in the sea urchin. In contrast with many others, he did not take an extreme view and maintain that development must be either mosaic or regulative. He interprets the experiments of Roux and Driesch as proving that:

> In normal development each of the blastomeres is profoundly influenced by the other; that the cell is not an isolated

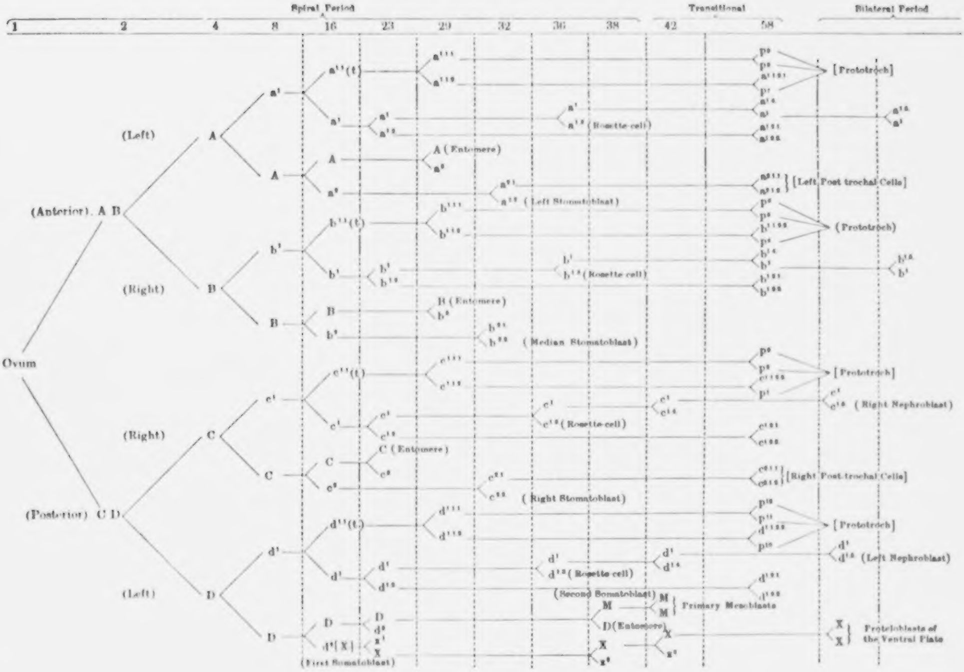
Spiral Period
Transitional
Bilateral Period
1
2
4
8
16
23
29
32
36
38
42
58
Ovum
(Anterior). A B
(Posterior) C D
(Left)
(Right)
(Right)
(Left)
A
B
C
D
[Prototroch]
(Rosette cell)
A (Entomere)
(Left Stomatoblast)
[Left Post trochal Cells]
(Prototroch)
B (Entomere)
(Median Stomatoblast)
(Right Nephroblast)
C (Entomere)
[Right Post trochal Cells]
(Right Stomatoblast)
(Left Nephroblast)
(Second Somatoblast)
Primary Mesoblasts
D(Entomere)
(First Somatoblast)
Proteloblasts of the Ventral Plate

FIG. 40. The cell lineage of *Nereis*. (Wilson, 1892, p. 382.)

> mechanism whose mode of action is wholly predetermined in its molecular structure. It proves in fact that the form of cell-division is determined by two factors. The first factor is the inherited tendency of the cell to pursue a definite course, a tendency which we may assume exists by virtue of a corresponding molecular or protoplasmic structure. The second factor is the influence upon the cell of other cells in the colony. When the second factor is removed or modified, the first is correspondingly modified, and a complete readjustment takes place. I can see no logical halting point in the application of this principle (p. 447).

In thinking about Wilson's conclusions remember that when he wrote there was almost no useful knowledge of either inheritance or cell physiology.

Wilson took a strong stand against the Roux-Weismann hypothesis of qualitative nuclear division, having tested it by subjecting unsegmented eggs of *Nereis* to pressure in order to obtain abnormal distributions of the nuclei (1900, pp. 411–412).

> If unsegmented eggs be subjected to pressure . . . they segment in a flat plate, all of the cleavages being vertical. In this way are formed eight-celled plates . . . . If they are now released from pressure, each of the cells divides in a plane approximately horizontal, a smaller granular micromere being formed above, leaving below a large clear macromere . . . . The sixteen-cell stage, therefore, consists of eight deutoplasm-laden macromeres and eight protoplasmic micromeres (instead of four macromeres and twelve micromeres, as in the usual development). These embryos developed into free-swimming trochophores containing eight instead of four macromeres . . . . In this case there can be no doubt whatsoever that four of the

> entoblastic nuclei were normally destined for the first quartet of micromeres, from which arise the apical ganglia and the prototroch. Under the conditions of the experiment, however, they have given rise to the nuclei of cells which differ in no wise from the other entoderm-cells. Even in a highly differentiated type of cleavage, therefore, the nuclei of the segmenting egg are not specifically different, as the Roux-Weismann hypothesis demands, but contain the same materials even in the cells that undergo the most diverse subsequent fate.

SPIRAL CLEAVAGE AND HOMOLOGY

Most embryologists in the 1890s, even the experimentalists, were still influenced by the Haeckelian paradigm and Wilson sought to relate his study of *Nereis* to studies on other embryos with spiral cleavage. At the very same time that he was working at Woods Hole another Hopkins graduate, E. G. Conklin was there studying cell lineage in a mollusk, the limpet *Crepidula*. The two of them made the astonishing discovery that the details of early cleavage in the annelid worm *Nereis* and the mollusk *Crepidula* were nearly identical. In both the three quartets of micromeres came off in the typical pattern of spiral cleavage: the first quartet clockwise, the second counterclockwise, and the third clockwise. But the truly startling discovery was that both formed a d^4 cell from which all mesodermal structures are derived in later development.

It is hard not to conclude that annelids and mollusks, phyla that differ so widely in the structure of their adults, retain some "ancestral reminiscences" (as Wilson, 1898, later called them) in the details of their early development. The concept of homology seems to apply.

Wilson pointed out (1892, pp. 439–443) that not only do some annelids and mollusks have the same pattern of spiral cleavage but so does a platyhelminth, the polyclad *Discocoelis*.

> Up to a late stage in the spiral period (twenty-eight cells) every individual blastomere and every cell-division is represented by a corresponding blastomere and a corresponding cell-division in the embryo of the polyclade, and in that of the gastropod [*Crepidula*].

So, if identity of patterns of cleavage can be taken as a criterion of homology, this would again appear to be a clear case.

But if one asks another question—is the origin of the mesoderm the same—the answer is not obvious. Figure 41 shows the cleavage pattern of annelids, mollusks, and polyclads. First note the great similarity of D, a mollusk, and E, an annelid. The three quartets of micromeres have been given off and the four macromeres are shown at the bottom. The important thing to notice is the origin of the mesoderm—shown as the shaded cell, M or d^4. The polyclad is shown in C and it is obvious that the pattern of cleavage is similar to that of annelid (E) and mollusk (D). In the polyclad, however, the origin of mesoderm, shown as shaded cells, is different.

> In the polyclade the first group of micromeres gives rise to the entire ectoblast, the second and third groups to the mesoblast, the macromeres to the entoblast. In the mollusk and annelid, on the other hand, the second and third groups of micromeres give rise to the ectoblast, like the first set, and the mesoblast arises subsequently. This remarkable divergence between the polyclade on one hand and the mollusk and annelid on the other is a fact of capital importance, for it proves that cells having precisely the same origin in the cleavage, occupying the same position in the embryo, and placed under the same mechanical conditions, may nevertheless differ fundamentally in morphological significance. We cannot escape the conclusion that the cell possesses a definite hereditary tendency upon which primarily its nature depends, however much its outward form or mode of division may be affected by the mechanical conditions of its environment in the body; and full weight must be given to this heredity in every attempt to interpret the origin and meaning of cleavage-forms (p. 441).

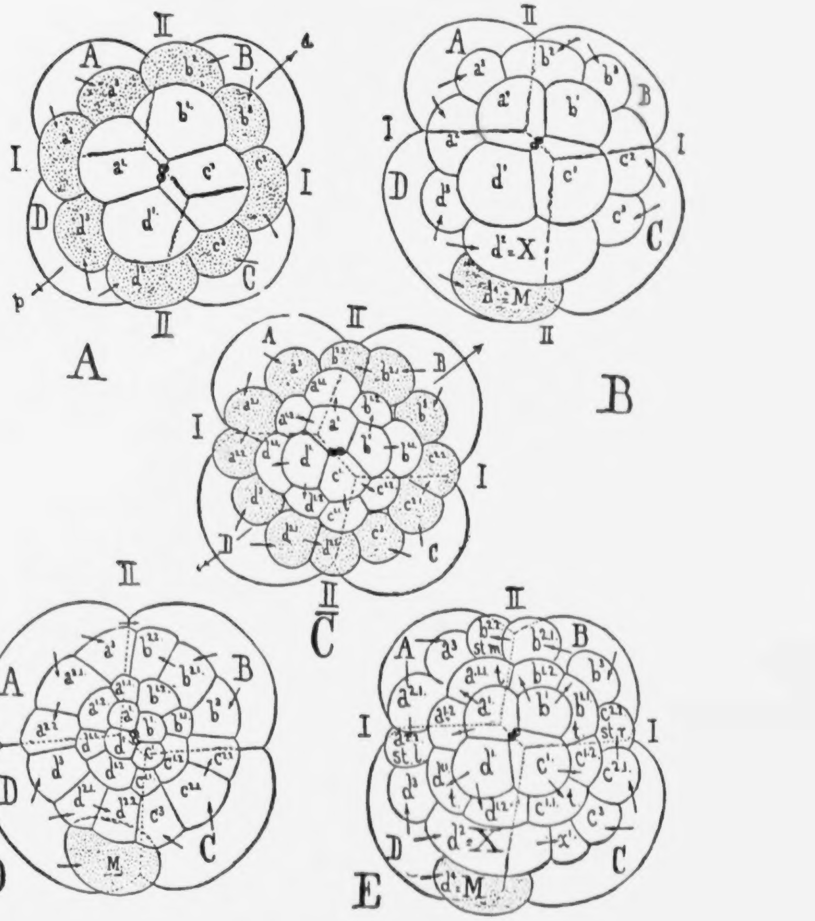

FIG. 41. Cleavage patterns in a polyclad (A, C), a mollusk (D), and annelid (B, E). Animal pole views. The cells that will form the mesoderm are shaded. In mollusks and annelids (D and E) the mesoderm is derived from a single cell, d^4, or M. In the polyclad (A and C) it is formed from the second and third quartets of micromeres. (Wilson, 1892, p. 440.)

What can one conclude, then, about the homologies of cleavage patterns and of the mesoderms? The answers depend, of course, on how homology is defined. If we define homology as the inheritance of the putative homologous structures from a common ancestor we will probably never know the answer—the likelihood of our being able to work out the early embryology of the presumed Precambrian ancestors is not promising. If we define homology as being strict identity of embryonic origin, we have to say that the mesoderm in polyclads is not homologous with the mesoderm in annelids and mollusks. That answer is not acceptable to many morphologists since there are other, and important, reasons for assuming that all mesoderms are homologous.

Wilson, always one to think deeply about the implications of his research and that of others, devoted one of the famous "Wood's Holl Lectures" (1895) to the embryological criterion of homology.

> The puzzling facts reviewed . . . leave no escape from the conclusion that embryological development does not itself afford at present any absolute criterion whatever for the determination of homology. Homology is not established through precise equivalence of origin nor is it excluded by total divergence . . . . But it does not by any means follow that the embryological method must be abandoned as a means of investigating homologies. The most skeptical critic of the recapitulation theory cannot deny that the embryological evidence is often of the clearest and most convincing character.

What, then, should the basic criterion be?

> Obviously it is the standard of Owen, viz., the structure and structural relations of the developed organs; it is the standard of comparative anatomy . . . . We must primarily take anatomy as the key to embryology, and not the reverse. Comparative anatomy, not comparative embryology, is the primary standard for the study of homologies, and hence of genealogical descent (pp. 113–114).

But can more be said about those embryological similarities that appear to indicate homologies? Wilson emphasizes that developmental stages do not remain unchanged in evolution but are capable of being modified much more than is generally thought. The fact that some aspects of development seem to be ancient is not surprising because:

> They point to the conclusion that the events of ontogeny are essentially adaptive, and that the persistence of ancestral reminiscences in development or of similarities in the development of homologous parts is in some way connected with the persistence of ancestral conditions of development (p. 121).

What should we conclude? It may be best not to make any strict conclusions about homology and merely note that, as in the examples given by Wilson, some members of the great phyla Mollusca, Annelida, and Platyhelminthes have a common pattern of spiral cleavage that apparently has an inherited basis. The most economical hypothesis is that it does reflect a common ancestry. In the case of the origin of the mesoderm, we can accept that in both annelids and mollusks it has a common and highly unusual embryological origin. Again, the most economical hypothesis is that it is a pattern inherited in common. The problem with the polyclads is more difficult. For many reasons it is useful to think of the mesoderm as homologous in the three phyla but that a genetic change has slightly altered the precise point of its origin in the polyclads—or alternatively that the polyclads represent the primitive condition and that it was the common ancestor of annelids and mollusks that underwent the genetic change.

In any event these difficult puzzles do have a possible answer as reflections of form and function in ancestors that lived at times so remote as to be nearly beyond human comprehension. Yet we do have a conceptual scheme that allows us to relate a variety of natural phenomena—a scheme that can be modified on the basis of new information and new hypotheses. Otherwise the commonality of spiral cleavages and d^4 cells is really not very interesting at all.

STYELA

One of the more remarkable cases of the visible organization of the uncleaved ovum and the early cleavage stages was provided by Conklin (1905). He had gone to the Marine Biological Laboratory at Woods Hole intending to study maturation of the egg and fertilization in the ascidian *Ciona intestinalis.* The adults proved difficult to obtain early in the season so he switched to two other ascidians, *Molgula manhattanensis* and *Cynthia partita* (now *Styela partita*), but quickly settled for the latter:

> The very first lot of the living eggs of *Cynthia* which I examined showed a most remarkable phenomenon and one which modified the whole course and purpose of my work; for there on many of the unsegmented eggs, which were of a slaty-gray color, was a brilliant orange-yellow spot, which in other eggs appeared in the form of a crescent or band. Further

> observation showed that this crescent became divided into two equal parts at the first cleavage and that it could be followed through the later cleavages and even into the tadpole stage. I therefore, for a considerable portion of the summer, devoted myself to the study of the living eggs of *Cynthia*.

And no wonder. Conklin had struck embryological gold and he was the careful and capable person worthy to develop the strike. He followed the changes from ovarian egg to fully formed larva. Only the early events will be described now (Fig. 42).

The mature oocyte has a large transparent germinal vesicle. The interior of the oocyte consists of a mass of gray yolk and the periphery contains a yellow pigment. When the germinal vesicle ruptures at the onset of meiosis, it liberates a quantity of clear material. At fertilization the sperm enters near the vegetal pole and this starts a dramatic rearrangement of the cytoplasm. The yellow cytoplasm (which appears black in the photographs of Fig. 42 of Conklin's beautiful colored plates) and the clear cytoplasm (from the germinal vesicle) flow toward the point of sperm entry where the yellow cytoplasm forms a peripheral cap and the clear cytoplasm is in the interior. This movement leaves the gray yolky material in the animal hemisphere where it surrounds the maturation spindle in the area where the polar bodies will form.

The yellow cytoplasm next moves to form an equatorial crescent extending about 180 degrees around the posterior part of the ovum. The clear cytoplasm moves toward the center of the ovum.

The first cleavage furrow bisects the yellow crescent. The clear cytoplasm and the yolky cytoplasm switch positions—so that the clear cytoplasm occupies the animal hemisphere and the yolky cytoplasm the vegetal hemisphere.

Conklin discovered that at the close of first cleavage these distinctively colored regions of the embryo have a precise relationship with the structures that would form subsequently. The fate of the yellow crescent is to form muscles and mesenchyme, the fate of the gray yolky cytoplasm is to form endoderm, and the clear cytoplasm of the animal hemisphere will form ectodermal structures. Conklin could even distinguish the area that would form the neural plate and the notochord (Fig. 43).

The striking aspect of these observations on the embryo of *Styela* is not that the positions of the structures-to-be are already fixed at the very beginning of development. The same is probably true for the frog's egg. *Styela* is notable because it has pigments that correspond to the boundaries of the germ layers that will form and this allows the embryologist to trace these areas in the course of development.

These studies of Whitman, Wilson, Conklin, and many others on cell lineage demonstrated that the mature ovum is a complex and highly organized structure. It is far from the isotropic cell hypothesized by Pflüger. Whitman had believed that although "we cannot say that the embryo is predelineated we can say that it is predetermined."

All one can really conclude from these studies, however, is that in the course of normal development identifiable regions of the very early embryo develop into specific structures of the older embryo. One cannot say that those regions can only form those structures of the older embryo. Neither can we say that the structures of the older embryo can be formed only by those delineated parts of the early embryo.

A careful distinction must be made between *fate* (prospective significance) and *capacity* (prospective potency). Fate means what an area of a younger embryo will form in a later embryo. Capacity means what the cells of that area of the younger embryo are able to do under a variety of experimental conditions.

The fate and capacity of an area of an early embryo may be the same. Such a situation would be where the region is irreversibly determined, that is, it self-differentiates into a specific later structure. Alternatively, that region of the early embryo might, under different conditions, have the capacity to produce much more than its normal fate would suggest, that is, it would have the capacity to regulate.

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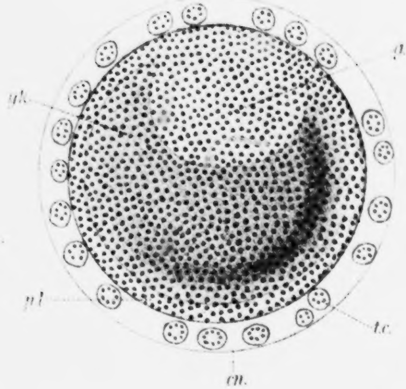
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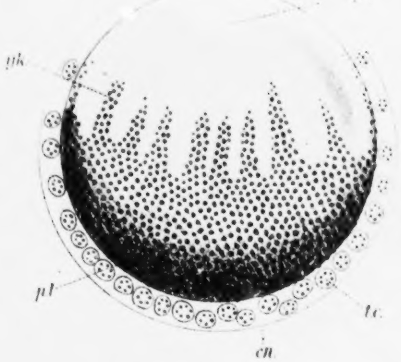
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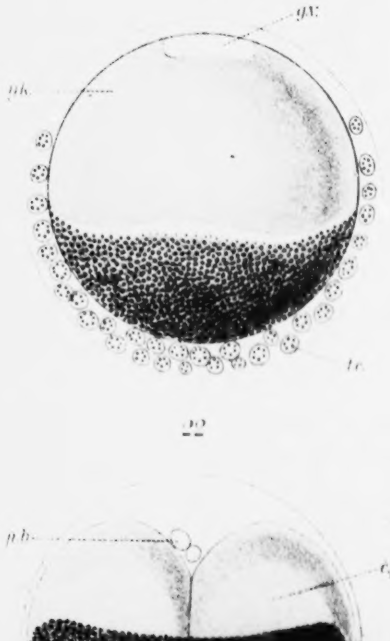
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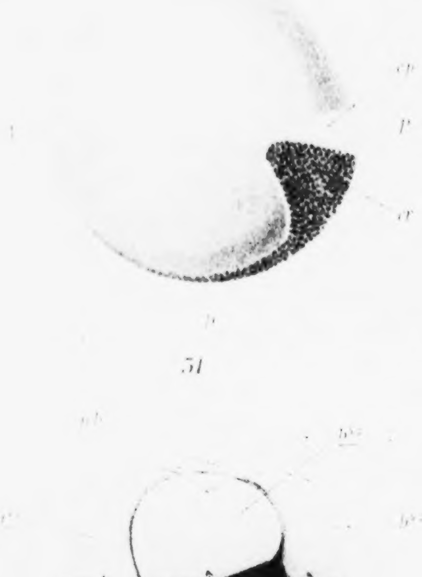
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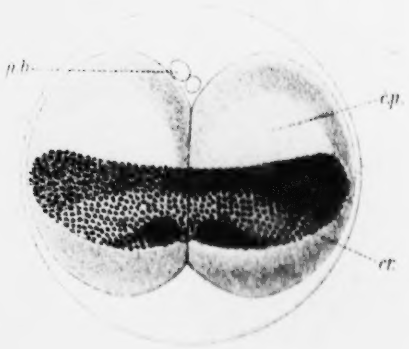
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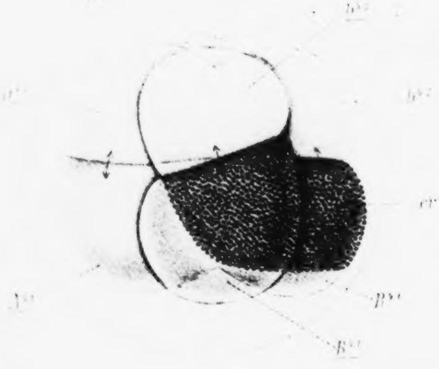
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cr.
A²
pv²
B²

Thus the distinction between mosaic development and regulative development, which has been applied to the whole embryo, must also be applied to the different regions of the embryo. Problems of this sort, and especially the determination of prospective potency, could be solved only by experimentation.

Embryologists undertook to isolate blastomeres and conduct other sorts of experiments intended to discover the interrelations of embryos and their parts. For example, what is the significance of the colored areas of the egg of Conklin's *Styela*? Does the yellow crescent represent the actual material necessary for the formation of mesoderm? Or is this colored area an indicator of the presence of other substances—the real organ-forming substances? Is the d^4 cell of *Nereis* and *Crepidula* the exclusive source of material required for the mesoderm to develop?

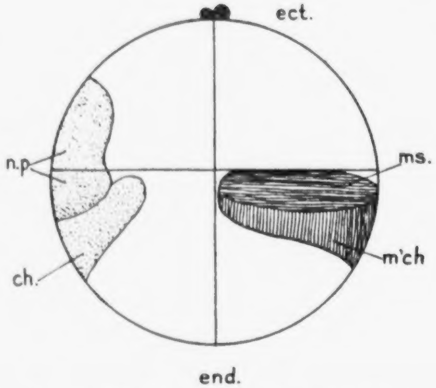

FIG. 43. Conklin's fate map for the ascidian embryo at the end of first cleavage. After the figure was drawn he realized that the presumptive chorda and the presumptive mesenchyme meet—hence, forming a complete equatorial band that separates the presumptive ectoderm above and the presumptive endoderm below. With this correction there is a striking resemblance of the fate maps of ascidian and amphibian as shown in Figure 25. (Conklin, 1905, p. 108.)

DENTALIUM AND *PATELLA*

The repertoire of techniques available to experimental embryologists at the turn of the century was limited and very crude. One could push hot needles into blastomeres or shake them apart. It was found that when cleavage stages of some marine invertebrates were placed in sea water without calcium ions the blastomeres separated, which meant that the isolation of blastomeres was made much easier. Simple hand centrifuges enabled one to stratify the more fluid parts of uncleaved eggs. It was discovered that some embryos could be cut with a scalpel.

Since there were not many experimental techniques available, embryologists adopted a strategy common in biology—search for organisms that differ from those that have already been studied in the hope of finding a new pattern of development—an experiment that nature had done—that might provide new information and new insights.

One interesting variant that nature provides is the presence of polar lobes in the early cleavage stages. Polar lobes are formed in many invertebrate embryos. They are non-nuclear structures that push out from blastomeres and then flow back into them. They are outgrowths of the vegetal hemisphere that appear to be a mechanism for redistributing cytoplasmic materials in the early cleavage stages.

Polar lobes are found in the embryos of the mollusk, *Dentalium*. Figure 44, from Wilson's classic study (1904*a*), shows the

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⟵

FIG. 42. The organization of the ascidian egg. These black and white photographs were made from Conklin's natural color illustrations. The pale yellow pigment of the living egg appears here as black. 1 is an unfertilized egg with the germinal vesicle (g.v.) beginning to break down; test cells (t.c.) are beneath the chorion (cn); the area shaded is the gray yolk (yk) and the egg is surrounded by a layer of clear protoplasm (p.l.). 3 shows an egg 5 minutes after fertilization; the yellow pigment and the clear protoplasm are collecting in the vegetal hemisphere. 4 shows the yellow pigment and the clear protoplasm entirely in the vegetal hemisphere. In 13 the yellow pigment and the clear protoplasm have formed crescents in the posterior part of the egg. First cleavage is underway in 22 and the crescents are bisected. 31 shows the eight-cell stage with the yellow crescent material restricted to the two posterior vegetal hemisphere blastomeres. (From Conklin, 1905.)

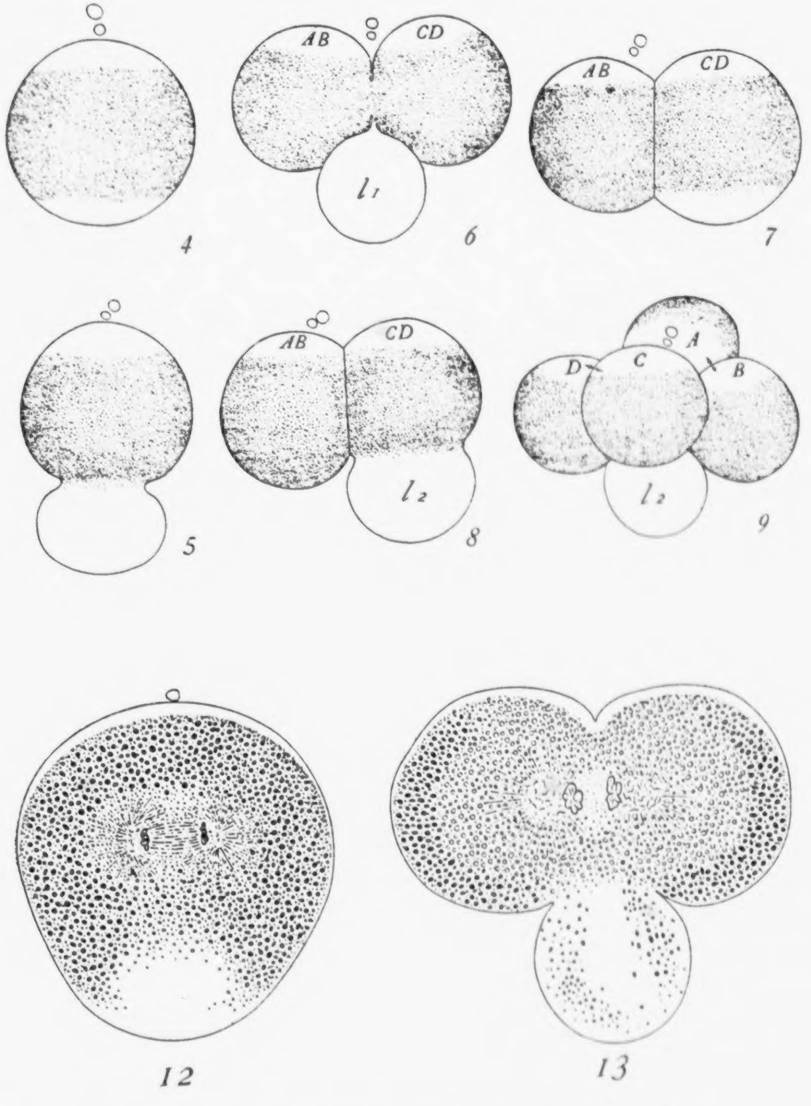


FIG. 44. Polar lobe formation in *Dentalium*. (Wilson, 1904*a*, pp. 6, 9.)

events up to the four-cell stage. When the eggs are shed from the ovary, they are divided into three zones: a clear cytoplasm at the animal pole, a central portion reddish in color, and another clear area at the vegetal pole. An embryo one hour after fertilization is shown in Figure 44, 4. The central pigmented area and the two clear areas are evident as are the polar bodies at the top.

Before first cleavage the first polar lobe forms at the vegetal pole, as shown in Figure 44, 5. It contains essentially all of the clear cytoplasm of the vegetal hemisphere. In embryo 6 cleavage is underway and the plane is such that the first polar lobe is

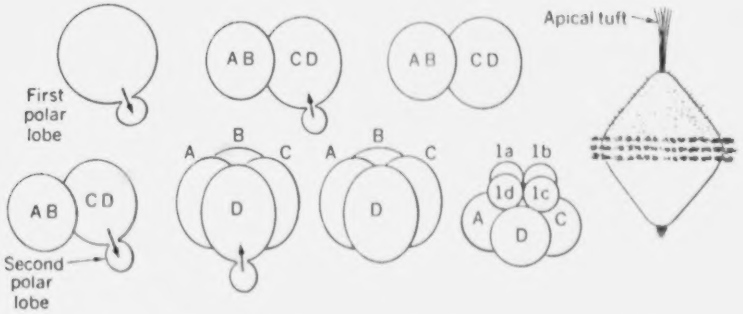


FIG. 45. *Dentalium.* Early cleavage stages and the trochophore larva.

attached to only one blastomere, called the *CD* blastomere. The first polar lobe is then withdrawn into *CD* and the completed two-cell stage is shown as embryo 7. Blastomere *AB* has clear cytoplasm only in the animal hemisphere but *CD* has it not only there but also in the vegetal hemisphere—the contents of the first polar lobe. As a result, *CD* is larger than *AB*.

A second polar lobe forms from the vegetal hemisphere of *CD*, as shown in embryo 8. Second cleavage occurs as in 9, and at its completion the contents of the second polar lobe are incorporated in the *D* blastomere.

Two drawings of fixed and sectioned embryos before first cleavage have been completed and are shown at the bottom of Figure 44. Embryo 12 has a thin cap of clear cytoplasm at the periphery of the animal hemisphere and the clear cytoplasm of the vegetal hemisphere is starting to form the first polar lobe. Embryo 13 shows the first polar lobe fully formed. The mitotic spindle is shown in both sectioned embryos—in the center of the egg, far removed from the polar lobe.

At third cleavage the *D* blastomere forms the third polar lobe, which then flows right back into *D*. Before this cleavage starts the clear cytoplasm near the animal pole moves clockwise and when the cells divide it becomes incorporated into the first quartet of micromeres.

These events are diagrammed in Figure 45.

> *In Dentalium the freshly discharged egg, prior to maturation or fertilization, shows a definite segregation of visibly different materials which accurately foreshadows a corresponding distribution of these materials among the blastomeres during cleavage* (Wilson, 1904*a*, p. 17).

Wilson underscored that sentence since it was describing, once again, the remarkable organization of the mature ovum.

EXCISING POLAR LOBES

A trochophore larva is formed in a day. It is top-shaped with an apical tuft of long, stiff cilia and an equatorial band—the prototroch—of three rows of motile cilia, a ciliated pretrochal region and a non-ciliated post-trochal region (Fig. 46, embryo 29).

Wilson sought to learn the significance of the polar lobes by cutting them off from the blastomeres with a scalpel and observing subsequent development. When he cut off the first polar lobe the second polar lobe failed to form. Otherwise the cleavages were normal. After 24 hours, however, a larva was formed that was a disaster (Fig. 46, embryo 32). It had three rows of prototrochal cilia that were larger than normal. The pre-trochal region is present—it can be identified by its covering with short cilia. The apical tuft is absent and so is the entire post-trochal region—the embryo ends at the prototroch. Embryo 29 is an unoperated control of the same age shown for comparison.

Embryo 36 (Fig. 46) had its second polar lobe removed. It also formed a larva with an exaggerated prototroch and no post-trochal region. It does, however, possess a

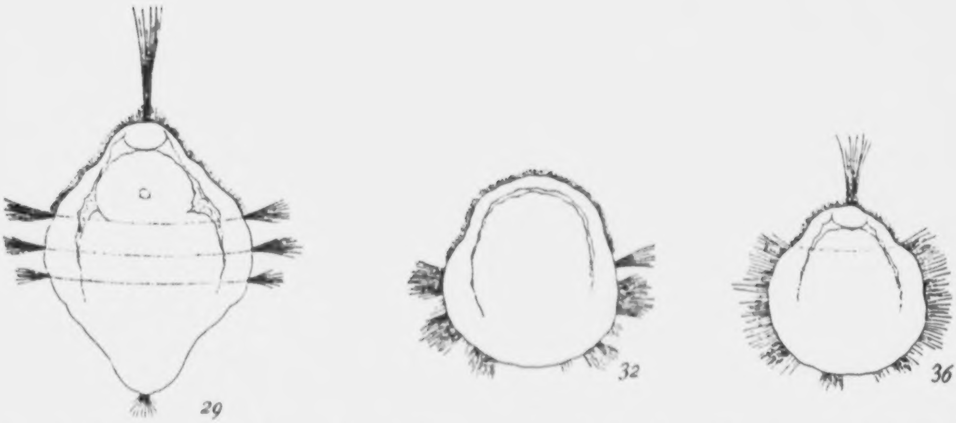


FIG. 46. Polar lobe elimination experiments. 29 is a normal trochophore larva. 32 is a larva that had its first polar lobe removed. 36 is a larva that had its second polar lobe removed. (Wilson, 1904*a*, p. 24.)

normal apical tuft. What sorts of hypotheses could your students propose for the determinants of the apical tuft and post-trochal region on the basis of the information so far?

Wilson was much impressed with the importance of the polar lobes especially since

> the amount of material removed with the polar lobe . . . is wholly disproportionate to the effect produced. The polar lobe includes less than one-fifth the volume of the egg; yet its removal does not merely cause a structural effect of like extent, but inhibits the whole process of growth and differentiation in the post-trochal region (pp. 56–57).

Figure 47 shows the results of Wilson's experiments in isolating blastomeres. His caption gives the details. When the blastomeres were cut apart at the two-cell stage the results were strikingly different. Embryos 45 and 46 are isolates from the same two-celled embryo. Embryo 45 developed from the *CD* blastomere and has an apical tuft, a ciliated pre-trochal region, a prototroch of normal size, and the nonciliated post-trochal region. Embryo 46 developed from the *AB* blastomere and is the same as embryos from which the first polar lobe is removed (Fig. 46, embryo 32).

Wilson then isolated blastomeres at the four-cell stage. Embryos 47 and 48 are both from a separated *CD* blastomere. Embryo 47, from the isolated *D* blastomere, is fairly normal, having both an apical tuft and a post-trochal region. Embryo 48, from the isolated *C* blastomere, is about the same as the isolated *AB* blastomere (embryo 46) or as an embryo from which the first polar lobe had been removed (Fig. 46, embryo 32).

Finally when he isolated the micromeres after the third cleavage, an important new bit of information was obtained. Embryo 49 (Fig. 47) developed from the 1*d* cell and embryo 50 from 1*c* of the same embryo. Only the 1*d* cell produces an embryo with an apical tuft.

With this additional information your students should be able to use these data and specify where the substances required for the apical tuft and the post-trochal region are localized. Wilson's experiments are splendid for asking questions of this sort: if removal of the first polar lobe results in a larva without the apical tuft or the post-trochal region, what would you predict would be the development of the isolated *CD* and *AB* blastomeres of a normal two-cell embryo (*i.e.*, one from which the polar lobe has *not* been removed!)?

Putting all these data together, Wilson

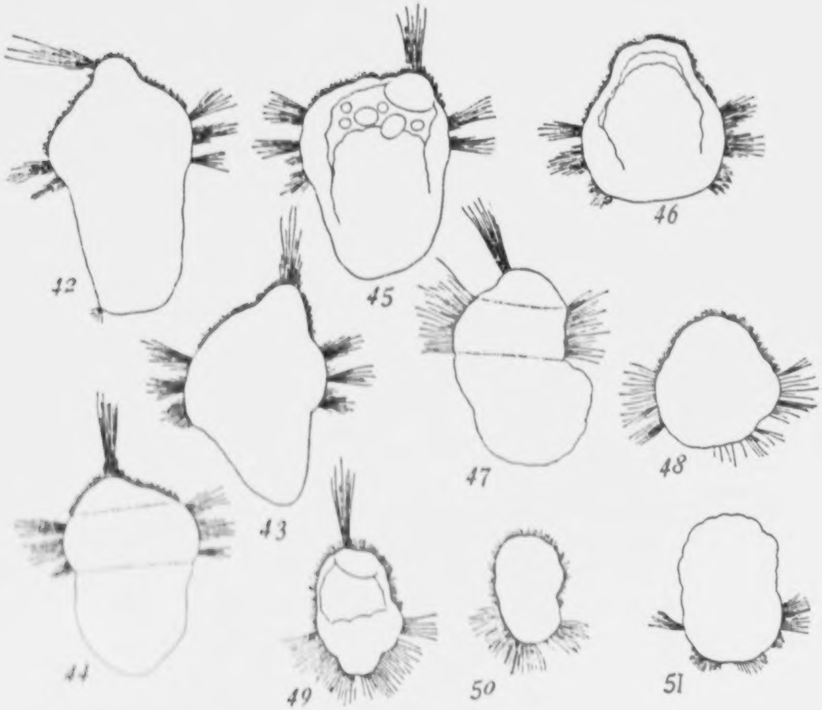


FIG. VII.

Larvae from isolated Blastomeres.

42, 43, 44, Various forms of larvae from isolated CD halves, 24 hours; 45, 46, twin larvae from the isolated CD and AB halves of the same egg, 24 hours; 47, larva from isolated D-quadrant, 24 hours; 48, larva from isolated C-quadrant of the same egg, 24 hours; 49, larva from isolated posterior micromere, 1d, of 8-cell stage, 24 hours; 50, larva from isolated micromere, 1c, of the same egg, 24 hours; 51, one-fourth larva from one of the small quadrants (A, B or C), 72 hours.

FIG. 47. Isolation of blastomeres. (Wilson, 1904*a*, Fig. VII.)

concluded that the substances in the egg that are necessary for the post-trochal region to develop are originally in the clear cytoplasm of the vegetal hemisphere of the uncleaved egg. They are then successively located in the first polar lobe, the *CD* blastomere, the second polar lobe, and finally in the *D* blastomere.

Similarly, the materials necessary for the apical tuft are first in the vegetal hemisphere, then successively in the first polar lobe, *CD* blastomere, *D* blastomere, and then the 1*d* micromere.

CYTOPLASMIC DETERMINATION

As noted before, the polar lobes do not contain a nucleus so the substances, or determinants, responsible for the apical tuft and post-trochal region must be cytoplasmic and they must be present in the egg before fertilization. Does this conclusion mean that the determinants are unrelated to genes? Almost certainly not. The hypothesis that will be developed later is that genes control the synthesis of the determinants while the ovum matures in the ovary. Essentially this conclusion was reached by Wilson more than three-quarters of a century ago.

> My observations demonstrate conclusively, I think, both the mosaic character of cleavage in these eggs, and the definite prelocalization of some of the most important morphogenic factors in the unsegmented egg. The *Dentalium* egg shows, even before it breaks loose from its attachment in the ovary, and long before even the initial changes of maturation, a visible definite topographical grouping of the cytoplasmic materials. This is proved by the experiments to stand in definite causal relation to the subsequent differentiation of the embryo in such wise that the removal of a particular cytoplasmic area [he had also cut off parts of eggs] of the unsegmented egg results in definite defects in the resulting embryo that are not restored by regenerative or other regulative processes within the time-limits of the experiment [*i.e.*, there was none of Roux's post-generation] (p. 55).
>
> The conclusion is therefore unavoidable that the specification of the blastomeres in these eggs is due to their reception, not of a particular kind of chromatin, but of a particular kind of cytoplasm; and that the unsegmented egg contains such different kinds of cytoplasm in a definite topographical arrangement (p. 56).

But his final conclusion is that all is ultimately under nuclear control:

> It therefore appears possible, not to say probable, that every cytoplasmic differentiation, whether manifested earlier or later, has been determined by a process in which the nucleus is directly concerned, and that the regional specifications of the egg-substance are all essentially of secondary origin (p. 64).

Wilson's experiments on *Dentalium* were done at the Naples Zoological Station between February and August of 1903, a period in his life when his long-held view of the importance of the nucleus, and specifically of the chromosomes, in inheritance—including development, of course—was prominent in his mind. His close friend Th. Boveri had recently published his experiments on dispermic sea urchin embryos, which showed that normal development depends on a balanced set of chromosomes (III, pp. 662–663). But more importantly, his student W. S. Sutton had just published his remarkable papers linking chromosomes and Mendelian inheritance (III, pp. 653–662). In a few years Wilson was to essentially abandon embryological work and devote his full energies to establishing the cytological basis of genetics (III, pp. 673–677). At the same time, Thomas Hunt Morgan, his colleague at Columbia University, would soon be making genetics an exact science (III, pp. 678–720).

MOSAIC DEVELOPMENT, REGULATIVE DEVELOPMENT—NEITHER OR BOTH?

If we ask "What were the Big Questions?" that concerned experimental embryologists during the last decades of the 19th century and the first one of the 20th, we will find that few were new. The dominant question was whether early development could be best described as mosaic or regulative. That was no more than an extension of the age-old debate over preformation *vs.* epigenesis. Studies of the organization of the mature ovum, the pattern of early cleavages, cell lineage, and the isolation of blastomeres were all designed to ascertain the degree to which the parts of the ovum were irreversibly determined, that is, irrevocably committed to a specific developmental pattern, or regulative, that is, with the capacity to do more than their normal fate would indicate.

These questions attempted to dissect the fundamental phenomena of development in order to understand differentiation better. They could not be answered by watching normal embryos develop. In a normal embryo the fates of the parts of an embryo and what the parts actually do are identical. The fate (prospective significance) of a part and the capacity (prospective potency) of that part, however, may differ widely. Capacity must be determined by subjecting the part to various abnormal situations.

Thus one cannot ask "What is the capacity of a single blastomere from the two-cell stage of an *Echinus* embryo?" and obtain an answer by watching development. All one could determine would be that half of an embryo produces half of a larva. However, if we isolate that blastomere, we learn that it has the capacity to do all that an entire embryo can do.

Tentative answers to these questions were obtained for the major groups of animals by the turn of the century. It was possible to describe embryos as being mosaic, regulative, or some mixture of these basically different patterns. These characterizations related to the early cleavage stages only since it was generally understood that, eventually, all embryos reached a mosaic stage where the parts would self-differentiate.

Wilson (1904*b*), in a companion paper to the one on *Dentalium* just considered, found that another mollusk, *Patella*, was also strictly mosaic. Together with these mollusks, the ctenophores, polyclads, and annelids were thought to be strongly mosaic. The amphibians and echinoderms were thought to be intermediate and amphioxus was regarded as the most regulative in the early cleavage stages.

As mentioned before, sometimes nature performed experiments for the experimental embryologists. For example, it seemed highly probable that *Homo sapiens* is a regulative species when it was realized that identical twins or identical triplets are derived from a single fertilized egg. That indicates that we are regulative at least up to the end of second cleavage.

Teratology provided other data. The frog embryo shown in Figure 48 had some developmental accident and ended up with two normal-sized heads. When this embryo was sectioned each head was found to have a normal brain, eyes, otic capsules, olfactory organs, and other head structures. Had this embryo been normal all of its cells would have produced some specific part. In the double headed embryo, however, some of those cells were channelled in a different direction—indicating that their capacity was greater than their expected fate.

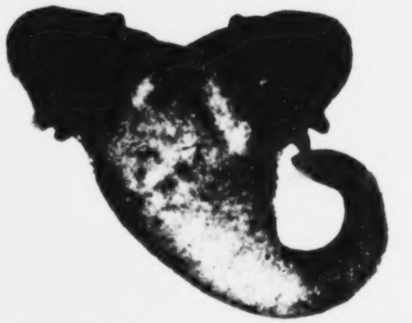


FIG. 48. A two-headed frog embryo. Spontaneously produced.

Before trying to make sense of this diversity there are two critical bits of information that, although of the greatest importance, are not usually emphasized. The first is that even the most regulative embryos tend to become mosaic at some stage in their development, as noted before. The second is that, in the regulative species even though one blastomere of the two- or even the four-cell stage can produce a normal larva, one is not justified in concluding that *any* half an egg can produce a whole embryo. Here nature might be misleading the egg shakers. In all eggs that had been studied it was realized that the unfertilized egg is organized to some degree. There was often a difference in pigmentation of animal and vegetal poles, frequently there was a gradient in the quantity of yolk granules in cells and, wherever it was possible to test, the polar bodies formed in a specific area of the ovum. (There is a third important fact that will be developed later, namely, that in even the most strictly mosaic species, their mosaicism is a transitory state—the annelid worms, for example, have remarkable powers of regeneration when they are adults.)

Thus it seemed beyond question that there are organized differences along the animal pole–vegetal pole axis. If the cleavage planes were at random with respect to the A–V axis, valid conclusions about the capacity of half-eggs, produced by a cleavage at any angle, could be drawn from the isolation of blastomeres experiments. But that is not what happens. The plane of the first cleavage is parallel to this A–V axis so the blastomeres are not receiving a random half of the contents when the egg divides.

All one can conclude from the development of isolated blastomeres of the two-cell stage is that a half-embryo cut by cleavage along the A–V axis will develop in a certain way. We cannot conclude that any half-embryo—such as one derived from an egg that cleaved horizontally to give an animal hemisphere cell and a vegetal hemisphere cell—will develop the same way. That notion occurred to those shaking the eggs apart. Driesch wondered if the *Echinus* egg had cleaved horizontally instead of vertically, would he have obtained the same result? He suspected the answer would be "no" and there were some data suggesting that answer. It was to remain for Hörstadius, a half century later, to provide the answer in some most elegant experiments.

In spite of all the variations among the embryos and vituperations among the scientists (some held firmly to the hypothesis that regulative development was the rule; others held firmly to the hypothesis of mosaic development), it did seem possible to provide a conceptual scheme to cover all embryos. By 1900 Wilson had developed such a scheme:

> The cytoplasm of the ovum possesses a definite primordial organization which exists from the beginning of its existence even though invisible, and is revealed to observation through polar differentiation, bilateral symmetry, and other obvious characters in the unsegmented egg . . . . [These] promorphological features of the egg are as truly a result of development as the characters coming into view at later stages. They are gradually established during the preembryonic stages, and the egg, when ready for fertilization, has already accomplished part of its task by laying the basis for what is to come (pp. 384, 386).
>
> In *Amphioxus* the differentiation of the cytoplasmic substance is at first very slight, or readily alterable, so that the isolated blastomere, as a rule, reverts at once to the condition of the entire ovum . . . . In the snail and ctenophore we have the opposite extreme to *Amphioxus*, the cytoplasmic conditions having been so firmly established that they cannot be readjusted, and the development must, from the onset, proceed within the limits thus set up.
>
> Through this conclusion we reconcile, as I believe, the theories of cytoplasmic localization and mosaic development with the hypothesis of cytoplasmic totipotence [and regulative development]. Primarily the egg-cytoplasm is totipotent in the sense that its various regions stand in no fixed relation with the parts to which they respectively give rise, and the substance of each of the blastomeres into which it splits up contains all of the materials necessary to the formation of a complete body. Secondarily, however, development may assume more or less of a mosaic-like character through differentiations of the cytoplasmic substance . . . . Both the extent and the rate of such differentiations seem to vary in different cases; and here probably lies the explanation of the fact that the isolated blastomeres of different eggs vary so widely in their mode of development. When the initial differentiation is of small extent or is of such a kind as to be readily modified, cleavage is *indeterminate* in character and may easily be remodelled (as in *Amphioxus*). When they are more extensive or more rigid, cleavage assumes a mosaic-like or *determinate* character, and qualitative division [of the cytoplasm], in a certain sense, becomes a fact (p. 423).
>
> It is important not to lose sight of the fact that development and differentiation do not in any proper sense first begin with the cleavage of the ovum, but long before this, during its ovarian history. The primary differentiations thus established in the cytoplasm form the immediate conditions to which the later development must conform; and the difference between *Amphioxus* on the one hand, and the snail or ctenophore on the other, simply means, I think, that the

> initial differentiation is less extensive or less firmly established in the one than in the other.
>
> [Thus] we reach the following conception. The primary determining cause of development lies in the nucleus, which operates by setting up a continuous series of specific metabolic changes in the cytoplasm. This process begins during ovarian growth, establishing the external form of the egg, its primary polarity, and the distribution of substances within it. The cytoplasmic differentiations thus set up form as it were a framework within which the subsequent operations take place in a course which is more or less firmly fixed in different cases (pp. 424–425).

The data available to Wilson supported the hypothesis that all eggs and even some embryos begin as highly regulative and then gradually become mosaic. The various species differ in the time when ovulation and fertilization occurs in relation to this transition from the regulative mode to the mosaic. That time comes early in amphioxus and late in *Dentalium* and *Patella*.

THE END OF AN ERA

When Wilson and others were reaching these conclusions, the next paradigm of experimental embryology was being formulated. It would be concerned not so much with the development of isolated parts of embryos as with the interactions among the parts. We will consider two examples: the work of Hörstadius on the sea urchin and that of the Spemann school on amphibian organizers.

Interest in the earlier problems did not cease, however. Old experiments were repeated with better techniques and better information. For the most part the results of the pioneers were confirmed but there was a general trend for finding the regulative eggs to be somewhat more mosaic and mosaic eggs to possess some regulative ability. The details can be found in a fine monograph edited by Reverberi (1971).

WORKING TOGETHER: *PARACENTROTUS*

During the 1920s and 1930s Sven Hörstadius, a Swedish experimental embryologist, performed a remarkable series of experiments on the eggs and embryos of the sea urchin, *Paracentrotus lividus.* He was skilled at operations on the minute embryos and his results, and their interpretation, are one of the main contributions to developmental biology in this century. He provided a fine summary of his work in 1973 (see also Hörstadius, 1939; Waddington, 1956; Giudice, 1973; Reverberi, 1971).

Only one aspect of this work will be considered here—the one showing that normal development requires the interaction of the parts of the embryo. This phenomenon is not encountered to any great extent in the mosaic eggs with their determinate cleavage and self-differentiating parts.

Echinoderm embryos have been favorite materials for experimental embryologists from the time of Driesch to the present. Mature *Paracentrotus* ova have a pigmented equatorial band that serves as a convenient landmark. As is true with so many species, the first two cleavages are meridional and the third is equatorial. The resulting eight cells are of approximately equal size (Fig. 49C).

The fourth cleavage, giving 16 cells, is vertical in the animal hemisphere, the result being a single layer of eight cells. The cleavage plane in the vegetal hemisphere is horizontal and unequal—resulting in four large macromeres and four small micromeres (Fig. 49D).

We will note the fifth cleavage only for what happens to the macromeres—they divide horizontally into two layers–called an_1 and an_2 (Fig. 49E).

Figure 49 is really a fate map for *Paracentrotus.* The boundaries of the cells, and their corresponding regions in the uncleaved egg (Fig. 49A), are differentiated so they can be traced throughout cleavage and up to the pluteus larval stage (Fig. 49M, N). The unbroken black line at the animal pole of the egg will become the an_1 layer. When traced through to an early larva (Fig. 49L) it is seen to form the ecto-

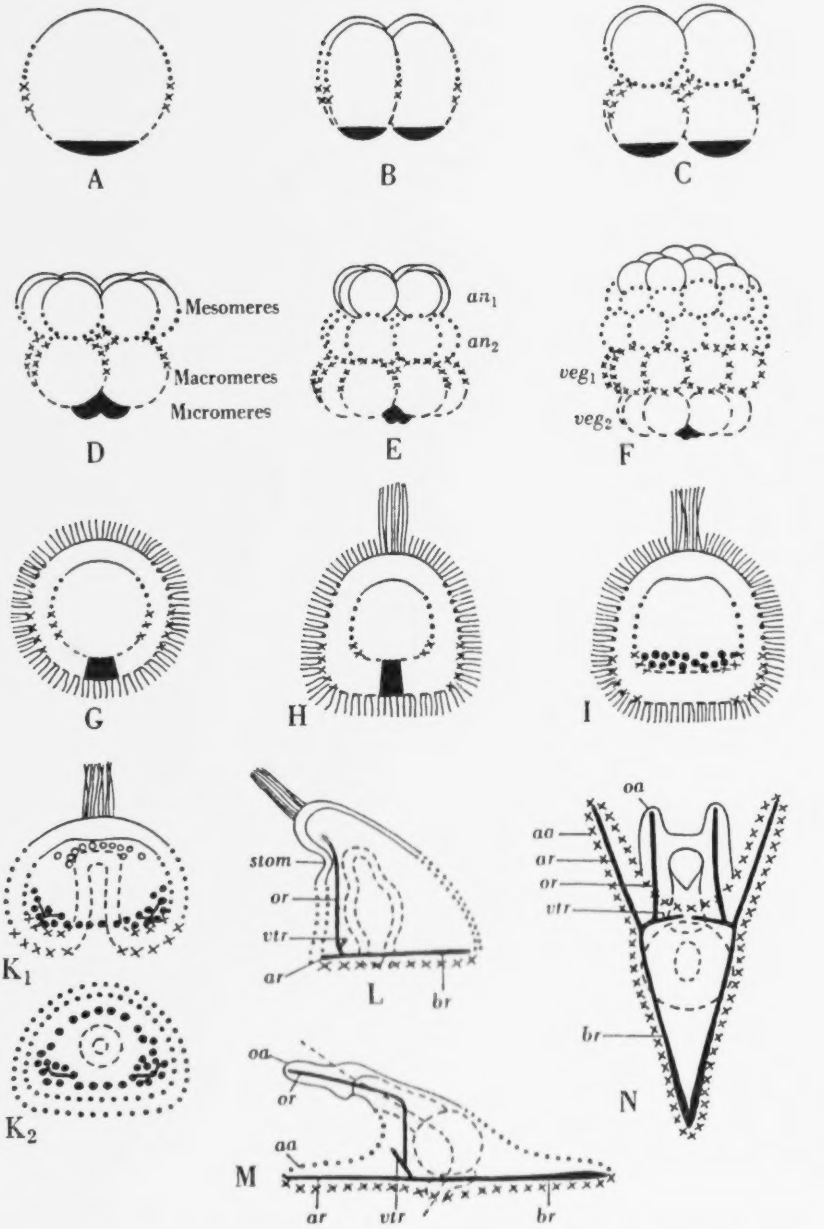

A
B
C
Mesomeres
Macromeres
Micromeres
D
an_1
an_2
E
veg_1
veg_2
F
G
H
I
K_1
stom
or
vtr
ar
L
br
oa
aa
ar
or
vtr
br
N
K_2
oa
or
aa
M
ar
vtr
br

FIG. 49. Normal development of the sea urchin embryo. See text for details (Hörstadius, 1939).

dermal covering of the upper part of the larva, the apical organ, and the stomodaeum.

The dotted lines show where the next layer of cells, an_2, come from and what their fate will be. It forms the epidermis (ectoderm) of the lower sides of the embryo (Fig. 49L). The parts of the early cleavage embryos that will form layer veg_1 are shown as crosses. This is also an ectodermal layer and its fate is to form the epidermis of the base of the early larva (Fig. 49L). Layer veg_2, shown in dashed lines, consists of the presumptive endodermal cells plus some mesoderm and it will form the archenteron, the secondary mesenchyme, and the coelom. At the region of the vegetal pole one finds the micromeres, shown solid black. Hörstadius reported that they form the primary mesenchyme and the larval skeleton (Fig. 49L, M, N).

The aspect of Hörstadius' work that forms an important part of our analysis is his experiments on separating embryos horizontally. This led to some important insights about development—showing that the egg and early embryo may have concentration gradients of animalizing and vegetalizing materials and, furthermore, that normal development is not so much a question of the parts involved but whether or not there is a proper balance of these two hypothetical types of substances. The animalizing substances are assumed to be necessary for the development of structures normally formed from the ectodermal areas. The vegetalizing substances are assumed to be necessary for the formation of those parts normally derived from the presumptive mesoderm and endoderm.

Hörstadius concludes that his data can be explained better by assuming that there are two gradients. Some investigators believe that the experimental data can be explained just as well by assuming one gradient of a single substance (Child, 1941, pp. 142, 240). We will assume that there are two.

The hypothetical animalizing substances are assumed to be in highest concentration at the animal pole and lowest at the vegetal pole. For convenience let us assume that they have a concentration of 5 in an_1, 4 in an_2, 3 in veg_1, 2 in veg_2 and 1 in the micromeres. In contrast, the vegetalizing substances are assumed to have the highest concentrations, let us say 5, in the micromeres and then decreasing one number per layer until they have a value of 1 in an_1. Let us also assume that normal development is possible only when the concentrations of the animalizing and vegetalizing substances are roughly equal in the whole embryo or fragment produced experimentally. Thus in a normal embryo, if we sum the values from an_1 to the micromeres, there will be a total of 15 animalizing units $(5+4+3+2+1=15)$ and the total will be the same for the vegetalizing substances $(1+2+3+4+5=15)$. (This system of giving arbitrary values to the hypothetical substances is not Hörstadius' but mine—it proved most helpful to students in introductory biology.)

The hypothesis that development is related to these substances can be tested by cutting the embryo horizontally between an_2 and veg_1 (Fig. 49F). The animal hemisphere half would have $5+4=9$ animalizing units and $1+2=3$ vegetalizing units. That ratio of 9 animalizing to 3 vegetalizing units is far from equal. The vegetal hemisphere half would have 6 animalizing and 12 vegetalizing units.

The results of such an experiment are shown in Figure 50. The upper row illustrates the blastulae derived from the animal hemisphere halves (consisting of an_1 + an_2—both presumptive ectoderm). When the blastula stage is reached, the apical organ, instead of being of normal size (Fig. 49H), may be expanded to cover nearly the entire embryo. The embryos A_1 through A_4 show the range of results, the majority being like A_1 or A_2. The A_4 type usually arises from embryos where the cleavage plane is somewhat lower than usual and so includes some of the material that would normally be in veg_1. The second row shows the limits of development of the animal halves. Most show no signs of gastrulation but those derived from A_4 may show slight invaginations, as in A_8.

The bottom row shows the development of the lower half (veg_1 + veg_2 + the micromeres; that is, one layer of ectoderm, one mainly of endoderm, and the micromeres, which form the primary mesenchyme and the skeleton). These plutei usually have an enlarged gut, poorly developed arms or none at all, and often no mouth. Earlier they usually lacked the apical organ.

These results seemed to support the hypothesis. The half with the hypothesized

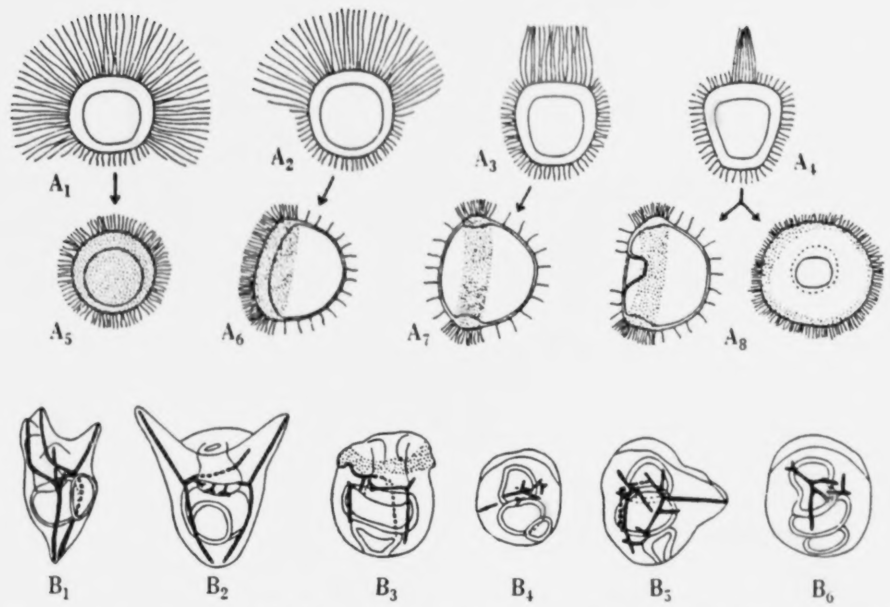


FIG. 50. Development of isolated animal (A's) and vegetal (B's) hemispheres of sea urchin embryos. See text for details (Hörstadius, 1939).

high level of animalizing substances and low level of vegetalizing substances does produce an animalized embryo unable to gastrulate. The vegetal half with the presumed high level of vegetalizing substances and low level of animalizing substances does seem to have exaggerated vegetal-type developments.

Such vegetalized embryos were not new. Since the turn of the century it has been known that, when normal echinoderm embryos are raised in sea water to which a small amount of a lithium salt has been added, they produce abnormal embryos with exaggerated vegetal structures.

Hörstadius' gradient hypothesis was tested in many other ways. He developed the techniques to separate the individual layers at either the 32- or 64-cell stages and combine them at will. These are some of the results (with our hypothetical values for the animalizing and vegetalizing materials in parentheses).

1. $an_1 + an_2$ = blastula with large apical tuft; almost never any gastrulation (9 animalizing and 3 vegetalizing units). Figure 51, A_1.
2. $an_1 + an_2 + veg_1$ = apical tufts normal but almost never any gastrulation. These three layers consist of the entire ectoderm (12 animalizing and 6 vegetalizing units). See Figure 51, B_1.
3. $an_1 + an_2 + veg_2$ = normal apical tuft. Reasonably normal pluteus larva (11 animalizing and 7 vegetalizing units). Figure 51, D_1.
4. $an_1 + an_2 + veg_1$ + micromeres = normal development (13 animalizing and 11 vegetalizing units). Figure 51, E_1.
5. $an_1 + an_2$ + micromeres = normal development (10 animalizing and 8 vegetalizing units). Figure 51, F_1.

Thus there is normal development when the ratio of animalizing to vegetalizing substances are close: 13/11, and 10/8; an intermediate condition when the ratio is 11/7; and abnormal development when the ratios differ markedly: 9/3 and 12/6. (If one desires, the values assumed for each layer can be adjusted so that normal development occurs when the sums of the animalizing and vegetalizing substances are approximately equal.)

Note that in experiment 3 the addition

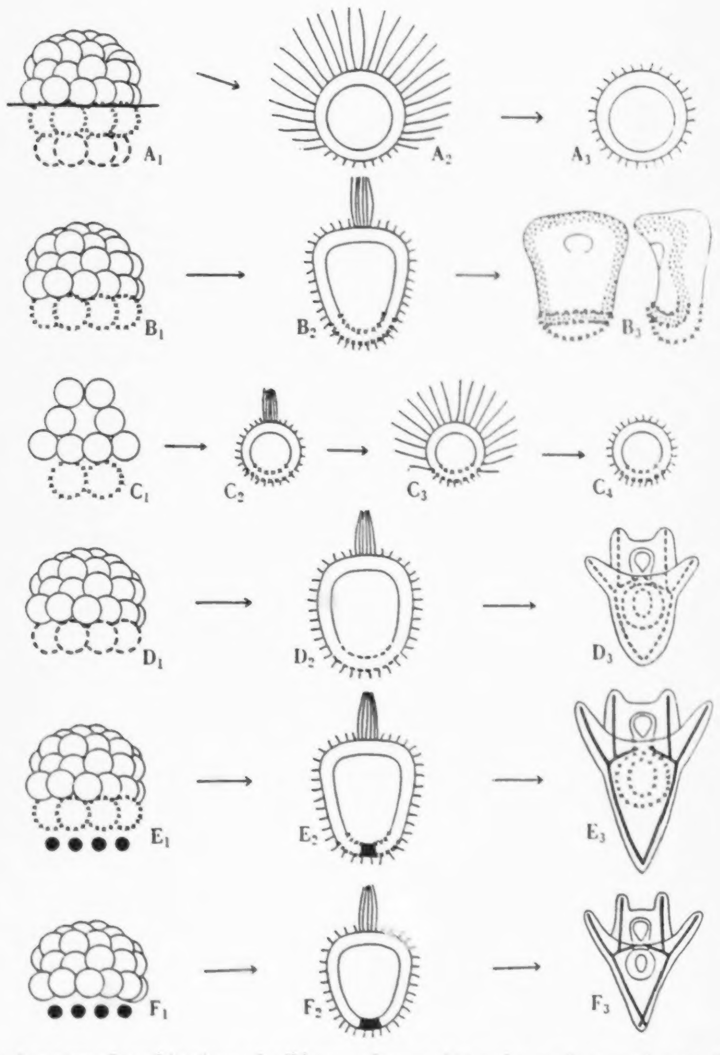


FIG. 51. Development of combinations of cell layers of sea urchin embryos. See text for details (Hörstadius, 1939).

of a single vegetal hemisphere layer, veg_2, is enough to balance the animalizing influences. A fairly normal pluteus is obtained even though a third of the presumptive ectoderm (veg_1), and the micromeres that normally form the primary mesenchyme and the skeleton are missing. Other cells, however, are able to alter their normal fates and produce the structures that the missing layers would have formed in a normal embryo.

Experiment 4 shows a similar result. The layer that would normally form the endoderm, veg_2, has been removed. The embryo remaining consists only of the presumptive ectoderm and the presumptive primary mesenchyme. Nevertheless an archenteron is formed.

The embryos of experiment 5 are much the same, except that they have lost a third of their presumptive ectoderm.

These results, plus many more not listed, lead to many important conclusions:

1. Earlier experiments by Driesch and

others on the isolation of blastomeres suggested that, although the pattern of cleavage and blastula formation indicated that the sea urchin embryos were partially mosaic, yet normal pluteus larvae were obtained indicating that the early embryos could regulate. The conclusion, therefore, was that half-embryos have the capacity to produce whole larvae. However these half-embryos were all obtained the same way—separation of blastomeres along the plane of first cleavage.

2. But now we find not just *any* half will suffice. When the blastomeres of the two-cell stage are isolated each will have the entire range of substances that are localized along the A–V axis. However, if the half-embryo is obtained by an equatorial cut, isolating the animal hemisphere and the vegetal hemisphere, as in the experiments just described, development is abnormal.

3. Thus the experiments on the isolation of halves show that the sea urchin embryo is mainly of the regulative type when the separation is along a meridian plane (the animal pole–vegetal pole axis) but largely mosaic when the separation is along an equatorial plane.

4. Although development can be explained by assuming concentration gradients of substances distributed along the A–V axis, these substances are not localized to specific areas. Any one of the five tiers of cells—an_1, an_2, veg_1, veg_2, and the micromeres—can be eliminated and a normal larva result. *Thus the development of a part depends on the entire embryo.* That is, the development of the part is regulated in such a manner that the end result is as normal as the entire fragment will permit. This hypothesis can be traced back to the late 1800s and it expresses the view of those who accepted the hypothesis of regulative development. Hertwig, writing in 1893, expressed it thus:

> Since every elementary part (*i.e* cell) arises through the division of the germ, or fertilized egg, it contains also the germ of the whole, but during the process of development it becomes ever more precisely differentiated and determined by the formation of cytoplasmic products according to its position with reference to the entire organism (blastula, gastrula, etc.) (quoted from Wilson, 1900, p. 415).

There are many morals to be learned from this research on sea urchin embryos. Probably the most important for students is that the "facts" of science are to be accepted only for the precise phenomena they are assumed to describe. Sea urchin embryos were the model for regulative development, a "fact" based on the development of halves obtained by the separation of blastomeres along the meridian extending from animal pole to vegetal pole. This "fact" is replaced by a better "fact" when experiments produce halves by isolation of animal and vegetal hemispheres.

We no longer can describe sea urchin embryos as "regulative" or "mosaic" but must specify which conditions and which parts are being discussed.

Driesch was not wrong; his statements were incomplete. Since the questions he asked were fundamental to our understanding of development, others sought to repeat his experiments. When they used his techniques they usually obtained his results. Hörstadius was able to ask the question in a different way and he obtained a different answer that expanded our understanding of early development.

Another moral: the test of a single deduction rarely establishes an hypothesis as "true beyond all reasonable doubt."

THE THEORY OF AMPHIBIAN ORGANIZERS

In the early 1920s a new paradigm began to attract notice. This was the line of work started by the German embryologist Hans Spemann (1869–1941), which sought to discover how the parts of an embryo influence one another. This led to the hypothesis that one part of an embryo, the *organizer*, can influence the differentiation of another part, the *reacting tissue*.

The hypothesis of organizer action was tested in many ways, with the embryos of many species, and by many experimenters. The hypothesis was abundantly confirmed and, since it accounts for a great variety of developmental phenomena, we can promote it to a "theory."

THE FORMATION OF THE NEURAL TUBE

The cells of an amphibian embryo in the late blastula stage are essentially the same throughout the entire embryo. To be sure there is a gradient of increasing size, with the smallest cells at the animal pole and the largest at the vegetal pole. There is also a gradient in the concentration of yolk granules, with the least number in the cells at the animal pole and the most in those at the vegetal pole. The animal hemisphere cells are packed with melanin granules whereas those of the vegetal hemisphere are relatively pigment free. Apart from these differences there is nothing to suggest the widely divergent destinies of the cells of different regions.

The conversion of the single-celled zygote into the many-celled late blastula is brought about by cleavage with little or no visible differentiation of the cells: they just get smaller. During gastrulation the cells become rearranged and the sites of the three presumptive germ layers can be located (Fig. 25). This recognition of germ layers, however, is based almost entirely on the location of the cells and not their appearance. Subsequently the slow process of cellular differentiation results in visibly different cell types—muscle cells, leucocytes, neurons, gland cells—that form the tissues and organs of the embryo.

The first system developed in an amphibian embryo is the nervous system, so it is not surprising that it engaged the interest of embryologists. Although in the interior of the adult, it appears on the outside of the early embryo. At the end of gastrulation a flattened area, the neural plate, becomes visible—extending anteriorly from the closed blastopore. Neural folds appear at the edges of the neural plate, move to the center, and fuse along their crests, forming a tube that lies under the outer epidermis (Figs. 20, 21, 33).

When embryos of these stages are examined in sectioned material, we find that by the time the neural plate is forming gastrulation movements have brought a sheet of presumptive notochord cells into a position below the neural plate (Figs. 27–31).

Repeated observation would show that these events always occur in normal development—as Vogt's fate map indicates (Fig. 25). The neural tube forms in a constant way with respect to the positions of the blastopore, archenteron, and polarity of the embryo. These constant relations must be important because, if something always happens in the same way, it is assumed that it is a fixed phenomenon, presumably with cause-effect relationships.

Thus our problem is to understand how, at the end of gastrulation, those presumptive ectodermal cells that are in the area above the roof of the archenteron become the neural tube, whereas the rest of the presumptive ectodermal cells, which look identical, becomes the epidermal covering of the body—brains *vs.* skin are very different fates. In our own case the difference is quite spectacular. One set of presumptive ectodermal cells becomes so changed that it can think about the epidermis; the epidermal cells can never think about the brain at all.

HYPOTHESES, DEDUCTIONS, TESTS

So we ask: "Why these different fates?" The answer can only come from experimentation but, as usual, there is that awesome problem of knowing what to do—that is, how to ask a question that is answerable. The first thing we might try is to ask those questions of the 1890s once again—"Is the part mosaic or regulative?" Two alternative hypotheses to explain how presumptive neural tube cells of the early gastrula become the neural tube suggest themselves.

Hypothesis 1. The presumptive neural tube cells of an early gastrula possess an inherent capacity to form neural tissue. They are determined, that is, they have within themselves all that is necessary to differentiate into a neural tube.

Hypothesis 2. The presumptive neural tube cells of an early gastrula do not possess an inherent capacity to form neural tissue. That is, they are still in a regulative stage and

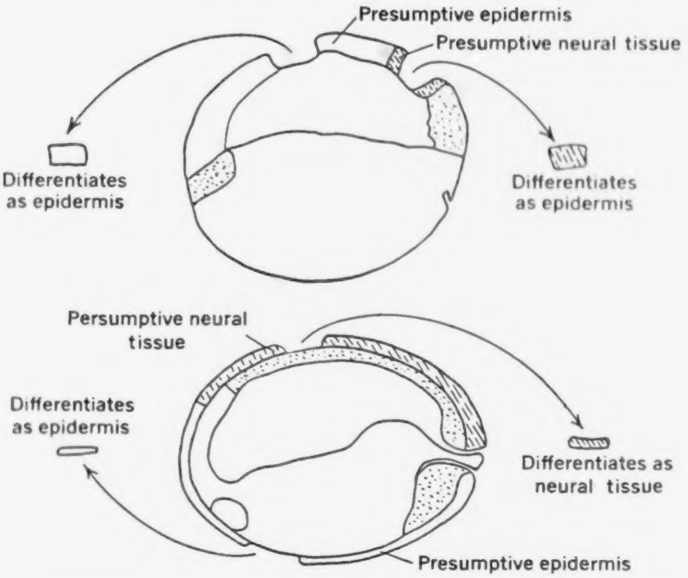

Presumptive epidermis
Presumptive neural tissue
Differentiates as epidermis
Differentiates as epidermis
Persumptive neural tissue
Differentiates as epidermis
Differentiates as neural tissue
Presumptive epidermis

FIG. 52. Explantation of presumptive neural tissue and presumptive epidermis in an early (above) and late (below) gastrula. Refer to Figures 27 and 30 for full labels.

> *influences from outside the presumptive neural tube area are necessary for them to differentiate into a neural tube.*

We can start by testing the first hypothesis. That is, we will provisionally accept that hypothesis 1 is true, make deductions, and then test the deductions.

If the presumptive neural tube cells are already determined and possess within themselves all that is necessary for the differentiation of a neural tube, this deduction follows logically:

> The presumptive neural tube cells should be able to differentiate into a neural tube if they are separated from the remainder of the embryo.

We now have to devise experimental means of verifying or denying the deduction. One such experiment was performed by Johannes Holtfreter (born 1901), a student of Spemann. Pieces of the blastocoel roof of an early gastrula are cut out and cultured in a dilute salt solution. No external source of food is required since each cell has many yolk granules. Such *explants* remain alive for days—many more than are necessary for the control embryos to gastrulate and form the neural tissue. Explants were taken from two areas—the presumptive neural tube area and the presumptive epidermis. The experiment is shown in Figure 52, top diagram.

The results of many experiments were the same: neither type of explant differentiated as neural tissue. Both formed only simple epidermal-like cells.

If these results can be accepted as an adequate test of the deduction, we must conclude that hypothesis 1 has not been supported. The experiment can be criticized, of course, as having resulted in injury to the excised piece of the blastocoel roof. This possibility can be partially ruled out since self-differentiation by other explants is possible, as we will soon see.

The evidence from this first experiment suggests that the presumptive neural tube cells have not been determined by the onset of gastrulation, since they are unable to self-differentiate. Nevertheless, they must become determined within a day because at that time they do form a neural tube.

Holtfreter now did the experiment at the end of gastrulation but before there was any indication of the neural plate. This experiment is shown in the bottom diagram of Figure 52 and the results were dramatically different: neural tissue was formed.

What could be the cause? The cells of the presumptive neural tube explant were older at the end of gastrulation, they contained fewer yolk granules, and they were smaller. They also were in a different environment. In the first experiment, before being explanted, the outer surface of both explants faced the outer environment while the inner surface faced the blastocoel. In the second experiment done at the end of gastrulation, before being explanted, the presumptive neural tube cells were above the presumptive notochord cells whereas the presumptive epidermal cells were above the presumptive endoderm. This might be significant.

Holtfreter found, quite by accident, another way to test hypothesis 1. In some experiments designed for an entirely different problem, early gastrulae were placed in water to which extra salts had been added, then the membranes surrounding the gastrula were removed, and the embryos were rotated so the animal hemisphere was down. Under these conditions gastrulation movements were abnormal. The presumptive ectoderm cells did not move down over the vegetal hemisphere but tended to pull away from the rest of the embryo. The result was a dumbbell-shaped embryo known as an exogastrula. In extreme cases the presumptive ectodermal cells formed an irregular mass connected by only a thin strand of cells with the presumptive endodermal and mesodermal cells.

A diagrammatic representation of the differentiation of these exogastrulae is shown in Figure 53. Development of the two parts was very different. The presumptive endoderm and presumptive mesoderm differentiated into heart, muscle, parts of the alimentary canal, and other organs normally formed from these two layers. These layers were able to self-differentiate. In marked contrast, the presumptive ectoderm remained essentially undifferentiated. There was no trace of a nerve tube.

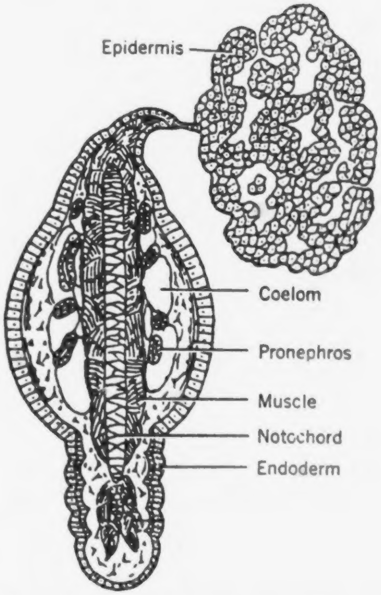


FIG. 53. The differentiation of the parts of an exogastrula (Holtfreter, 1933*b*, p. 406).

Exogastrulae are strange not only in this separation of presumptive ectoderm from the other regions but in the abnormal movements of the other two presumptive regions. The embryo turns inside out. As a result the mesoderm is inside the endoderm and the *lining* of the archenteron faces outward (Fig. 53).

Once again, the data indicate that the presumptive neural tissue is undetermined at the onset of gastrulation. We know, however, that it is determined by the end of gastrulation. Thus some change must occur in the interval between the early gastrula and the late gastrula. This change, however, does not occur in the presumptive neural tube tissue during the time it is an explant or part of an exogastrula—its cells do not become determined. We might suspect, therefore, that the change is due to influences from other parts of the embryo; and this would mean almost certainly influences from either the presumptive mesoderm or presumptive endoderm, or both.

That fits our second hypothesis, which implies that the presumptive ectoderm is completely undetermined at the onset of gastrulation and that some outside influence results in part of it being determined to become neural tissue. Since only part of the presumptive ectoderm becomes neural tissue, the stimulus from outside must be localized. If this is the case, the following deduction can be made:

> If the relative positions of the animal hemisphere, which contains the presumptive ectoderm, and the rest of the embryo are altered, the position of the neural tube should be altered accordingly.

An experimental test of this deduction was made by Spemann. He cut off the upper part of the animal hemisphere of an early gastrula, rotated it 180°, and stuck it back on the lower portion of the embryo. The two parts healed and the embryo went on to form a normal larva, not in relation to the presumptive regions of the animal hemisphere but of the ventral part.

Figure 54 shows the experiment. The upper figures are normal, unoperated embryos. The fate of the presumptive ectoderm is shown. As Vogt had established, the presumptive notochord area (stippled in the figure) is above the dorsal lip and the presumptive neural tube above that. The lower two figures show the operation. The animal hemisphere was cut along the dashed line and then rotated. As a consequence, the presumptive neural tube area is now 180° from its normal position and the presumptive epidermis is adjacent to the presumptive notochord. The operated embryo continues to develop but the neural folds appear in their normal position *with reference to the dorsal lip of the blastopore*. This means that the presumptive epidermis formed the nerve tube and the presumptive neural tube cells formed epidermis!

This experiment shows that the differentiation of the presumptive ectoderm is greatly influenced by the ventral part of the embryo. But what part? The constant relation of the dorsal lip of the blastopore to the position of the neural plate and neural tube, both in normal development and in the experiment on rotation of the animal hemisphere, suggests that the dorsal lip might be involved. The dorsal lip is the place where the presumptive notochord cells turn in, forming the archenteron roof, and come to lie beneath the presumptive neural plate. Recall that in the first experiment (Fig. 52) the presumptive neural tube tissue became determined after the presumptive notochord cells moved under it to form the archenteron roof.

These experiments and their analysis suggest a variation on hypothesis 2.

> *Hypothesis 2a. The presumptive neural plate cells of an early gastrula do not possess an inherent capacity to form neural tissue. Instead, the presumptive neural plate cells become determined as a result of stimulation by the presumptive notochordal cells of the archenteron roof.*

If this hypothesis is accepted as true, the following deduction can be a test of it.

> If the dorsal lip cells are removed from a donor embryo and grafted into a host embryo, and if they are able to invaginate, a nerve tube should be produced from the overlying presumptive ectoderm of the host.

This difficult (at the time) experiment was performed in 1924 by Hilda Mangold, when she was a student of Spemann. It is one of the classics of embryology, winning a Nobel Prize for Spemann in 1935 (Hilda Mangold had died shortly after the experiments were performed).

The operation is shown at the top of Figure 55. In order to recognize the origin of the cells, embryos of two species of salamander were used. In one species the embryos are nearly white and in the other they are brownish. A small piece of tissue was removed from the dorsal lip region of the donor embryo and then transplanted to a site 180° from the host's dorsal lip.

The host, therefore, had two dorsal lips—its own and the donor's. Invagination occurred at both. Because of the difference in pigmentation of the two species, it could be established that the dorsal lip cells of the donor invaginated. At the time the host's neural folds were forming (the

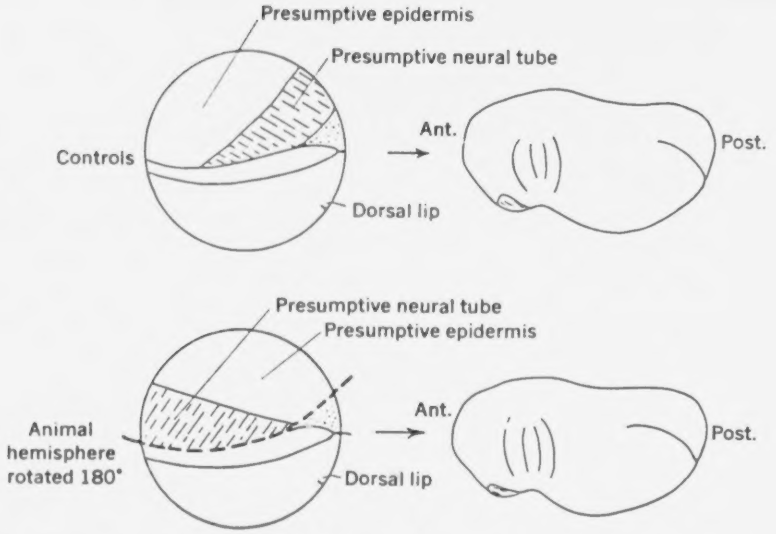


FIG. 54. Experimental rotation of the animal hemisphere. Refer to Figure 25 for full labels.

primary embryo), neural folds also appeared above the region where the donor dorsal lip cells had invaginated (the secondary embryo). The sectioned embryo is shown at the bottom of Figure 55. The secondary embryo is essentially normal.

An important question now confronts us. Is the secondary embryo formed from the donor tissue, host tissue, or both? Again, we can tell because of the difference in pigmentation of host and donor tissue. The answer is both. The donor tissue forms the archenteron roof of the secondary embryo, which later becomes the notochord. It also forms other structures, mainly mesodermal. The neural tube, however, is formed almost entirely from host cells. Thus cells that normally would form epidermis now form a nerve tube.

Spemann and Mangold had shown that the presumptive notochordal cells that invaginate at the dorsal lip and form the roof of the archenteron have a profound effect on development. They spoke of these cells as the *organizer* and their action on the undetermined ectodermal cells as *induction.* Induction is not restricted to events in only the secondary embryo but is a phenomenon of normal development.

The experiments so far described suggest that in normal embryos the neural tube is formed under the influence of the organizer. At the beginning of gastrulation, the organizer region consists of the cells above the dorsal lip corresponding roughly to the presumptive notochordal region of Vogt's fate map (Fig. 25). This region invaginates to form the roof of the archenteron. The roof of the archenteron then induces the overlying ectoderm to form a neural tube. Without this inductive influence these cells will form only simple epidermis.

We now have a theoretical basis to interpret the experimental results from explanation of tissues, exogastrulation, and the rotation of the animal hemisphere.

When presumptive neural plate cells from an early gastrula are explanted, they will never be induced by the organizer and hence cannot form neural tissue.

The same is true of exogastrulae—the presumptive ectoderm is never in contact with the organizer. In this case, however, Holtfreter made some most interesting observations. He found that by varying the culture conditions he could obtain partial exogastrulae. In these instances the presumptive ectoderm that was in contact with the presumptive mesoderm and endoderm was induced to form neural tissue.

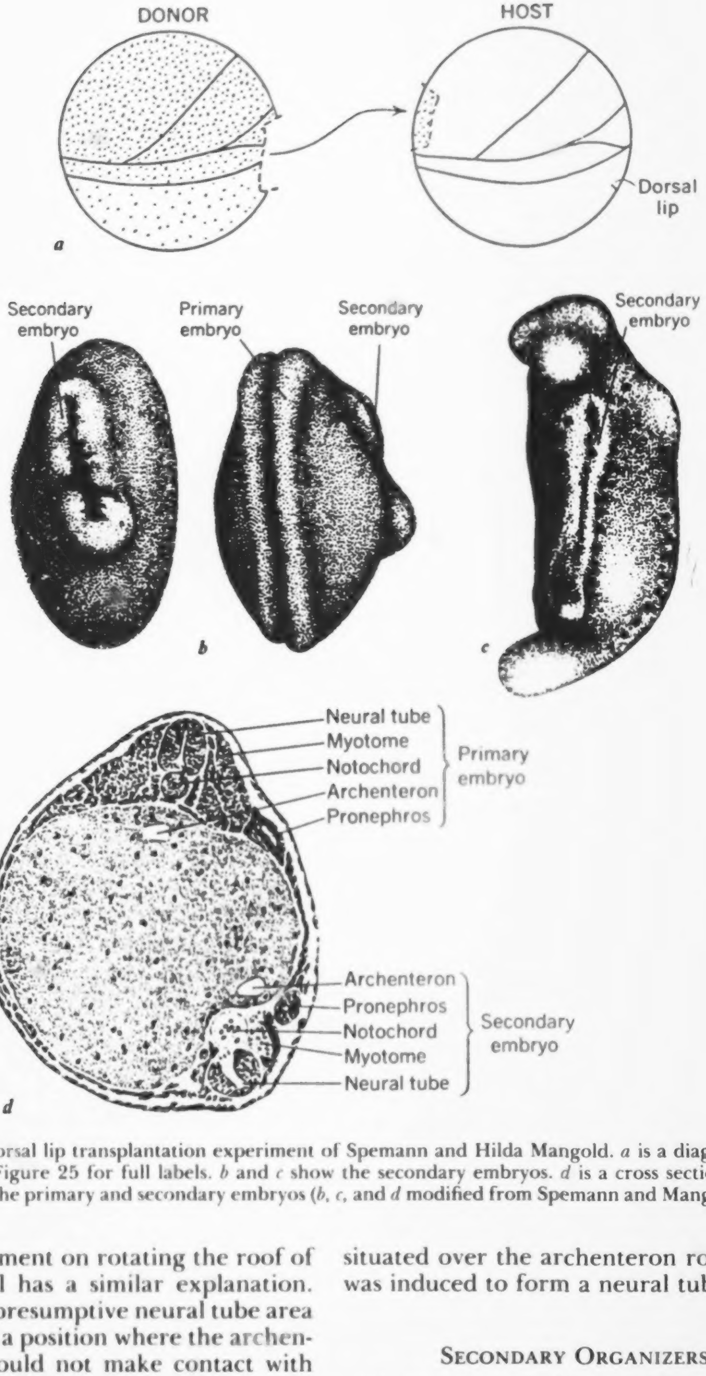

DONOR
HOST
Dorsal lip
a
Secondary embryo
Primary embryo
Secondary embryo
Secondary embryo
b
c
Neural tube
Myotome
Notochord
Archenteron
Pronephros
Primary embryo
Archenteron
Pronephros
Notochord
Myotome
Neural tube
Secondary embryo
d

FIG. 55. The dorsal lip transplantation experiment of Spemann and Hilda Mangold. *a* is a diagram of the operation—see Figure 25 for full labels. *b* and *c* show the secondary embryos. *d* is a cross section showing the structure of the primary and secondary embryos (*b*, *c*, and *d* modified from Spemann and Mangold, 1924).

The experiment on rotating the roof of the blastocoel has a similar explanation. The original presumptive neural tube area was moved to a position where the archenteron roof would not make contact with it—and it remained as epidermis. The presumptive epidermis, however, came to be situated over the archenteron roof and it was induced to form a neural tube.

SECONDARY ORGANIZERS

It was soon found that there is not just one organizer, the one associated with the tissue of the dorsal lip which later forms the archenteron roof, but many. Organizers were discovered for the mouth, heart, eye, lens, otic vesicle, olfactory organs, pronephros, and many other structures. (In fact there is much evidence to suggest that the primary axial organization of the early embryo is controlled by the invaginated material that forms the walls of the archenteron.) Secondary organizers act subsequently to but in the same manner as the primary organizer, that is, undetermined cells of the embryo are induced. Once these cells are determined they can self-differentiate.

The formation of the optic cup and lens of the eye can serve as an example.

THE FORMATION OF THE EYE

Vogt's fate map (Fig. 25) shows the amphibian eye as having a dual origin. The bottom diagram shows the presumptive optic cups in the middle of the presumptive neural tube area. The lenses, however, are the small ovals above, to the right and left, in the presumptive epidermis area. They are shown and labelled in the upper diagram.

The complete eye has the lens centered, which is of course necessary for normal vision. An off-centered lens would be useless. When we remember the complicated movements of the presumptive areas during gastrulation and neurulation, one can only marvel that the processes are so precise that the lens always ends up exactly where it should. But there is more to the story.

Shortly after the closure of the neural folds, the optic cups begin to grow laterally from the floor of the brain (Fig. 34). When the optic cup reaches the epidermis, a lens begins to form from the inner layer of the epidermis opposite the middle of the optic cup. Subsequently the outer layer of the epidermis, still full of pigment granules and quite opaque in Figure 34, begins to clear and form the cornea.

Experiments have shown that, in some species at least, the optic cup acts as an organizer that induces the head epidermis to form a lens.

The optic cup area itself seems to be induced by the archenteron roof. That is, the primary organizer not only induces the overlying ectoderm to form a neural tube but also induces a regional specificity.

The experiments designed to throw light on the formation of a lens are performed as follows. When the optic cups are beginning to form, a slit is made in the head epidermis and the optic cup on one side is cut off. The epidermis is pushed back in position and heals in a few minutes.

The embryo is allowed to develop for two days and then fixed and used for serial sections. The optic cup on the unoperated side (we have an experimental and control animal in a single individual!) is found to have produced a normal eye with lens. On the operated side, however, the brain is found to have healed and there is no optic cup at all. The brain cells, therefore, could not regulate to replace the excised optic cup. Of greater significance, however, is the fact that there is no lens on the operated side. Thus, in the absence of an optic cup, lens differentiation does not occur. This result suggests that the optic cup may be the organizer for the lens.

The next experiment supports that conclusion. The optic cup is removed when it is first starting to form and placed under the epidermis of the trunk region. The wound heals (amphibian embryos are just wonderful in this way) and, at the time a lens normally forms, the epidermis over the transplanted optic cup forms a lens. Thus it seems true beyond all reasonable doubt that, in the species used, the optic cup induces the overlying epidermis to form a lens. That trunk epidermis would normally have continued to differentiate as epidermis but this experiment shows that it still has the ability, or competence in the language of embryologists, to do more than its fate suggests.

Students are sure to ask about that eye back in the flank, which may appear to be entirely normal. "Does that eye enable the tadpole to see where it has been or, at least, who is sneaking up behind it?" No, the transplanted eye never makes the proper nerve connections. (This is a good place to reinforce the notion that we "see" with our brains, not our eyes.)

THE REACTING TISSUE

These descriptions of the induction of neural tubes and lenses have emphasized the role of the inducing agent. This may have given the impression that the reacting tissue is passively molded by the organizer. This is not the case. The ability of tissue to respond to organizers is limited in several ways.

Age is one limitation. The experiments described before showed that any portion of the presumptive ectoderm of an early gastrula can be induced to form a neural tube but this competence is short lived. At or about the stage when the neural folds close the presumptive epidermis is no longer capable of being induced by the archenteron-roof organizer. However, it is still competent to respond to other organizers—the optic cup, for example.

Tissue specificity is another limitation. The type of experimentation shown in Figure 52 has been extended to all parts of the early gastrula. Explantation is a test of the degree to which a tissue has been determined at the time of explantation and hence the extent to which it can self-differentiate. Such tests show that the presumptive ectoderm of an early gastrula has not been determined. If similar explantation experiments are done with the presumptive notochord and adjacent mesodermal regions of an early gastrula, another result is obtained. Both kinds of explants, although too small to produce organs, differentiate into notochordal, neural, and some other tissue types. These cells, therefore, are partially determined. They can form differentiated tissues but they are not completely determined—or they would form only what their fate suggests. There are problems with endodermal explants, as the cells tend to fall apart, but indirect evidence suggests that the presumptive endoderm is probably fully determined.

The ability of tissues to respond, their competence, can be tested in other ways. When small pieces of an early gastrula are transplanted to various parts of the body of an older embryo, such as a neurula, one discovers another important property of the reacting tissue. If pieces of presumptive ectoderm are transplanted, they are found to participate in the formation of whatever structure is present in the region where they are placed (Fig. 56). If transplanted to the heart region, heart tissue is formed; to the liver region, liver; to the kidney region, kidneys; to the brain region, brain. The same is true of the presumptive chorda-mesoderm as well.

The presumptive ectoderm and presumptive chorda-mesoderm, therefore, do not exhibit germ-layer specificity. Seemingly the cells of those regions do not know to which germ layers they belong.

Figure 57 summarizes the embryological state of the parts of an early gastrula. Much of the work is that of Holtfreter, who has been preeminent in adding to our understanding of amphibian development. His figure *a* is a fate map, essentially the same as Vogt's (Fig. 25). Note the special symbols for each presumptive area since they are repeated in *b* and *c*. Figure *b* shows the ability of explants from each region to self-differentiate. Figure *c* shows the capacity of the cells of each area to respond when transplanted to older embryos (as in the competence experiments shown in Fig. 56). The cells of most of the embryo can participate in the formation of any structure or tissue. Figure *d* shows the distribution of the dorsal lip organizer.

Genetic specificity is another limitation. The dorsal lip transplantation experiments of Spemann and Hilda Mangold involved two species of salamanders, then known as *Triton taeniatus* and *Triton cristatus*. The embryos of *taeniatus* are pigmented; those of *cristatus* are pale. These pigmentation differences can even be detected in histological preparations. Thus when a *cristatus* dorsal lip was transplanted to *taeniatus*, it was possible to say that the *taeniatus* ectoderm had formed the neural tube.

But which kind of neural tube? Was it a *taeniatus* neural tube or a *cristatus* neural tube? That is, does the structure of the induced neural tube conform to the species of the host or the species of the donor? That question cannot be answered, since the neural tubes of the two species are identical in shape and overall appearance. What is required is a system where the

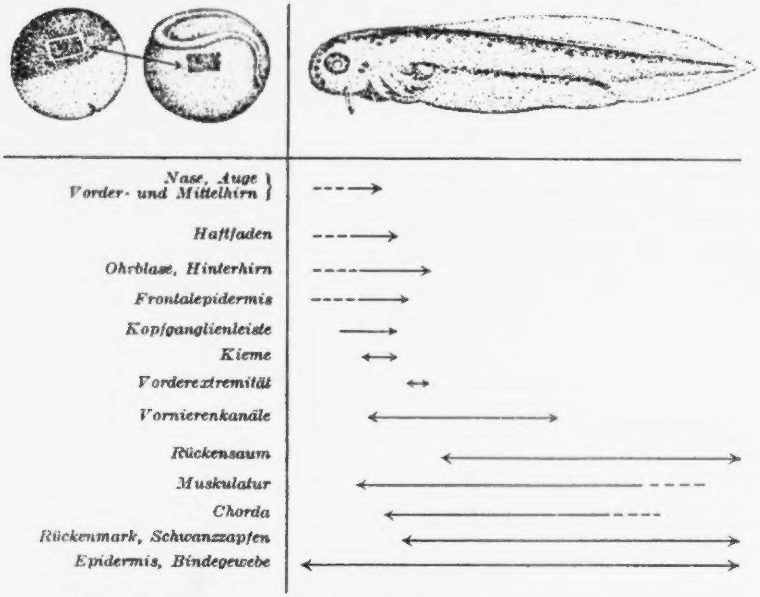


Abb. 68. Regionale Verbreitung der aus Gastrulaektoderm hervorgegangenen Differenzierungen auf dem Wirt (determinierende Felder der Neurula).

FIG. 56. Testing the competence of the presumptive ectoderm of an early gastrula. Gastrula tissue of a salamander was transplanted to various sites in an older embryo where it formed structures appropriate to the location in the host. The lines with arrows show the structures, listed at the left, that were induced in the donor tissue. Thus a line drawn directly down from the two spots just anterior to the larva's gills shows that the transplanted ectoderm can form olfactory organs, eyes, forebrains, midbrains, balancers, ears, hindbrains, frontal epidermis, neural crest, gills, pronephric ducts, muscles, epidermis, and connective tissue when placed at that site on the host. (Holtfreter, 1933*a*, p. 759.)

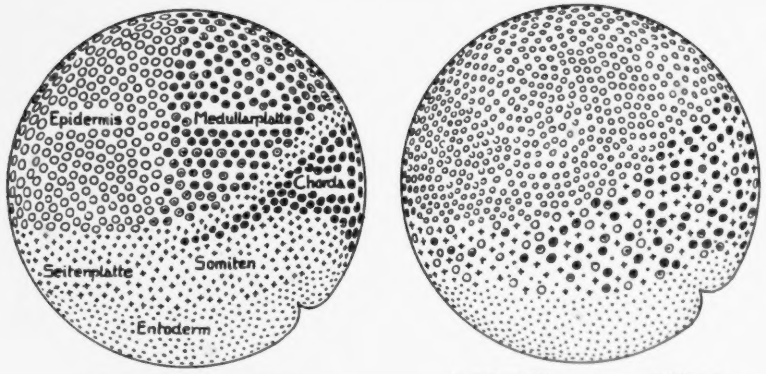
induced structure is recognizably different in host and donor.

Again nature supplied the material. The mouth regions of frog and salamander larvae differ greatly. The frog larval mouth is bordered by black, horny jaws and rows of tiny teeth (these are formed by the ectoderm and have no relation to the true jaws and teeth). The salamander larva lacks both ectodermal jaws and teeth; its mouth is just a hole in the head.

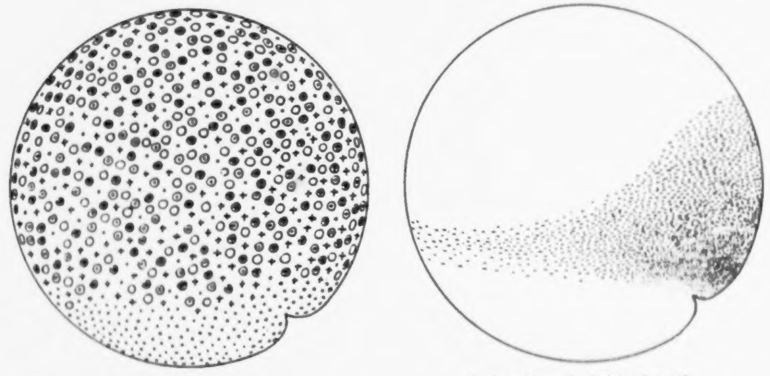
Since in young frog and salamander embryos it is possible to interchange the ectoderm of the region where the mouth will form, we have the prospect of answering the question: "If a mouth region is induced, will it be characteristic of the host or of the donor species?"

The results of such experiments are clear cut. The frog ectoderm on the salamander embryo is induced by the salamander mouth-region organizer to form a mouth. That mouth is of the frog type—with those horny jaws and teeth. In the reciprocal experiment the salamander ectoderm on a frog host produces a salamander mouth.

Other experiments of this sort have been tried and a general rule emerges: the tissue responds in accordance with its specific genetic constitution. Competent tissues can react to organizers but they must do so their own way. One is left with the impression that organizers are general stimuli and that the end result of their action is modulated by the genetic limitations of the reacting tissue. In normal embryos, of course, there is no problem—both the organizing tissue and the reacting tissue are from the same individual and hence have the same genes. It is only under



a Prospektive Bedeutung. b Selbstdifferenzierungsleistung.



c Prospektive Potenz. d Primärer Induktorbezirk.

Abb. 32 a–d. Schemata der Determinationsverhältnisse im frühen *Gastrula*-Stadium der Urodelen.

FIG. 57. The developmental state of early gastrula cells. *a* shows the fate, that is, the presumptive regions (compare with Fig. 25); the symbols repeat in *b* and *c*. *b* shows the ability of explanted bits of tissue from various parts of the gastrula to self-differentiate when explanted. *c* shows the capacity, or competence, of cells to form structures when transplanted to various regions of older embryos. *d* shows the distribution and relative potency of the primary organizer. (Holtfreter, 1936, p. 406.)

experimental conditions that unite inducing and reacting tissues of different genetic types that we uncover this principle of the limitation of the reacting tissue's response.

RECAPITULATION AGAIN

The extensive and detailed work on the experimental embryology of amphibians made important contributions to our understanding of recapitulation. For example, one can easily understand, why that "useless" structure, the notochord, forms in the amphibian larva only to be replaced by the vertebral column in the adult. The experimental evidence points to the vital role of the notochordal area or the roof of the archenteron in the induction of the central nervous system. Other experiments have shown that it plays the same role in all other vertebrates. The notochord, therefore, although of transitory importance as a skeletal element, is part of the basic organization of the vertebrate embryo. It is present in all vertebrates because it is necessary if the embryo is to get past the gastrula stage.

There is a similar group of experiments for the pronephros, which is also recapitulated in all vertebrate embryos. It is functional in the amphibian larva but is replaced by the mesonephros, which is the functional kidney of the tadpole and adult frog. Chick embryos start with a pronephros, but it is never functional. Their functional embryonic kidney is the mesonephros; and their adult kidney is the metanephros. Why bother with that useless structure, the pronephros? It turns out it isn't useless at all—when the pronephric duct is cut in either amphibian or chick, the mesonephros fails to develop. Like the notochord, the pronephros plays a vital, though brief, role in embryonic development even though it has no functional role in the adult.

The discovery of the inductive role of some recapitulated structures allows us to reevaluate that concept which was so puzzling, and so important, to 19th century embryologists and morphologists. There were serious disagreements but these were largely of our own making. Two fundamental errors were made: first, it was assumed that structures such as the notochord or the pronephros are "useless," and, second, that development and evolution were considered to be so demanding that inefficiencies would be rapidly eliminated by natural selection.

Both assumptions are at least partially wrong. Now we understand that some of those recapitulated "anomalies" are parts of the fundamental mechanisms of development. These mechanisms are built into the gametes under the direction of the parental DNA. The organization of the ovum, for example, will be what has proved successful for the lineage over time—success here being measured by survival and efficient reproduction. If the vertebrates early on evolved a system whereby the presumptive notochordal cells of the archenteron act as an organizer for the central nervous system, there is every reason for it to be preserved, not eliminated, by natural selection.

There is a pseudo-problem, however, of why it is necessary for the presumptive notochordal cells to actually differentiate histologically into a notochord. Why not just have those cells act as the organizer without going to the "trouble" of histological differentiation? An equally valid question is, "Why not differentiate as notochordal cells?"

This becomes a problem because of our second erroneous assumption: developmentally and evolutionarily this must be the best of all possible worlds. Yet we are wrong to assume that evolution must produce the most efficient patterns of development and adult life. Natural selection is stringent only to the degree that enables the species to "get by," or "good enough is good enough." Were this not the case we might be presumptuous enough to expect all evolutionary lineages to be leading to *Homo sapiens*, as was indeed believed by some pre-Darwinian evolutionists.

If we grant that the second assumption is erroneous, we should expect that some ancestral reminiscences would remain as part of the baggage of inherited developmental patterns. These would be structures with no detected importance in development, and theoretically some might be of no importance at all. They could remain because they are so innocuous that there are no selective pressures to eliminate them. Of course, we must remember that these ancestral reminiscences are the exception.

Our final conclusion: Structures *are* recapitulated and there are good reasons why they should be. It is largely the recapitulation as envisioned by von Baer—the sharing of a common pattern of development by the diverse organisms of a natural group. That being the case, it is inevitable that to some degree ontogeny gives the appearance of recapitulating phylogeny but, to an even greater degree, ontogeny recapitulates ontogeny.

The Nature of the Organizer

Clearly the dorsal lip organizer is of great importance in development, so not surprisingly there was eagerness to know what it was. Questions of this sort were asked in the 1930s when endocrinologists were discovering more and more hormones and were able to purify some of them. Could the organizer be a hormone-like substance? One could hypothesize that the roof of the archenteron might secrete a hormone-like substance that caused the overlying ectoderm to form a nerve tube?

The organizer was found to be widely distributed. The structures in other vertebrates—fish, reptiles, birds, and mammals—equivalent to the dorsal lip of amphibians acted as organizers when tested on amphibian embryos. This was exciting, but an even more exciting discovery was that pieces of the dorsal lip, or of the archenteron roof, could be killed by heat or chemical means and still induce undetermined ectoderm to form neural tissue. This was so important because it showed that the organizer was a stable chemical substance and that meant it might be possible to extract and purify the active principle.

But soon things started to get out of hand, or at least out of theory. Not only would dead dorsal lips induce but so would dead tissue from any part of an amphibian gastrula. Earlier experiments to determine the extent of tissue that could serve as an organizer had shown that such ability is restricted largely to the presumptive notochordal region and the presumptive endoderm above the dorsal lip in living embryos (Fig. 57). There was no organizing ability in living presumptive ectoderm but now, when killed, there was.

It was also discovered that tissues of many invertebrates, none of which possess a notochord or dorsal nerve tube, would also induce when killed. What was equally baffling was the finding that dead adult tissues, such as kidney or liver, could induce.

And the list became ever more bizarre: silica, kaolin, methylene blue, steroids, egg albumin, and polycyclic hydrocarbons were all found to have inductive power.

Some investigations suggested that these substances are not really organizers but are acting as toxic substances that somehow stimulate amphibian embryonic cells to form neural tissue. Although this is not a satisfying explanation, at the present time, there is none other.

There is no question that some tissues having no obvious relation to archenteron roofs are potent organizers. The liver of adult mice or guinea pigs, especially if treated with alcohol, can induce head structures in amphibian embryos. On the other hand, guinea pig kidney is a potent inducer of trunk structures.

The problem seems insoluble. There is simply no way at present to specifically identify the substance in the archenteron roof that causes the overlying ectoderm to form a neural tube if such a wide variety of other substances have the same effect. If one is searching for a substance, there must be some way of identifying it. The original test was the ability of the archenteron roof to induce neural tissue in competent ectoderm. But since essentially any tissue when killed will induce, one is left with no way of screening for the *real* organizer substance.

We must await new ideas and new techniques.

And what did its discoverer, Hans Spemann, think of the nature of the organizer? He was not even willing to think of it in chemical terms. This is how he concluded his monograph *Embryonic Development and Induction* (1938):

> There still remains, however, an explanation which I believe to owe the reader. Again and again terms have been used which point not to physical but to psychical analogies. This was meant to be more than a poetical metaphor. It was meant to express my conviction that the suitable reaction of a germ fragment, endowed with the most diverse potencies, in an embryonic "field," its behavior in a definite "situation," is not a common chemical reaction, but that these processes of development, like all vital processes, are comparable, in the way they are connected, to nothing we know in such a degree as to those vital processes of which we have the most intimate knowledge, viz., the psychical ones. It was to express my opinion that, even laying aside all philosophical conclusions, merely for the interest of exact research, we ought not to miss the chance given to us by our position between the two worlds. Here and there this intuition is dawning at present. On the way to the

> high new goal I hope to have made a few steps with these experiments (pp. 371–372).

That is from one of the most influential biologists of the early 20th century. It's hard to known what to say—except that apparently no one has followed his line of thought. Shades of Driesch!

References: Experimental embryology

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PUTTING IT ALL TOGETHER

Or can we? There has been a general feeling that developmental biology, almost alone among the biological sciences, is yet to achieve a satisfactory conceptual coherence.

A generation ago, Joseph Needham (1959, p. 240) observed that

> . . . strictly evolutionary dominance in embryology did not last on into the twentieth century. The unfortunate thing is that nothing has so far been devised to put in its place. Experimental embryology, Morphological embryology, Physiological embryology, and Chemical embryology form today a vast range of factual knowledge, without one single unifying concept, for we cannot yet dignify the axial gradient doctrines, the field theories and the speculations on the genetic control of enzymes, with such a position. We cannot doubt that the most urgent need of modern embryology is a series of advances of a purely theoretical, even mathematico-logical, nature. Only by something of this kind can we redress the balance which has fallen over to observation and experiment; only by some such effort can we obtain a theoretical embryology suited in magnitude and spaciousness to the wealth of facts which contemporary investigators are accumulating day by day.

Needham did not accept his own challenge—he went on to his magnificent project of chronicling the history of science and technology in China.

The Biological Revolution that started in 1952 with Watson and Crick had little immediate large scale effect on developmental biology. According to Medawar (1965):

> Embryology is in some ways a model science. It has always been distinguished by the exactitude, even punctilio, of its anatomical descriptions. An experiment by one of the grand masters of embryology could be made the text of a discourse on scientific method. But something is wrong; or has been wrong. There is no *theory* of development, in the sense in which Mendelism is a theory that accounts for the results of breeding experiments. There has therefore been little sense of progression or timeliness about embryological research. Of many papers delivered at embryological meetings, however good they may be in themselves . . . one too often feels that they might have been delivered five years beforehand without making anyone much wiser, or deferred for five years without making anyone conscious of great loss.
>
> It has not always been so. In the 1930's experimental embryology had much the same appeal as molecular biology has today . . . [this] was mainly due to the 'organizer theory' of Hans Spemann, the theory that differentiation in development is the outcome of an orderly sequence of specific inductive stimuli . . . .

But efforts to discover the chemical properties of the organizer failed and

> Wise after the event, we can now see that embryology simply did not have, and could not have created, the background of genetical reasoning which would have made it possible to formulate a theory of development.

These rather dismal opinions of developmental biology should be taken less as an evaluation of the field and more as an evaluation of working scientists. What is already known to them, no matter how magnificent, is of lesser interest than the tantalizing unknown. For the working scientist the great discoveries are valued more for guiding new research than for synthesizing that of the past. This has been true for Darwinian evolution, Mendelian genetics, Morgan's genetics, and the capstone set by Watson and Crick.

Therefore, no matter what the discovery, there is always a burning need to understand biological phenomena at a more basic level. Reductionism is so powerful a force in biology that we tend to overlook the fact that we may have satisfying explanations at one level even though there is always more to be discovered at more "basic" levels. The discovery that blood circulates has proved to be a sufficient answer, even to this day, for many physiological and medical questions. The discovery of the heart's pacemaker enriches the basic discovery—it does not make it less adequate. Similarly we can accept the knowledge that light, expressed as day length, affects the breeding behavior and migration of birds without having to know whether light consists of particles, waves, or both.

I suspect that we know more about developmental biology than we are willing to admit and that Horder (Horder *et al.*, 1986, p. xvi) may have a wiser vision:

> The meaningless of this question ["What is Life?"] to us now makes one wonder what equivalent pseudo-questions may be influencing our priorities today. It may well be that the very idea of 'the unsolved problem of embryology' is one such; the phrase itself almost presupposes a particular form of solution and invites particular forms of research, tending to invoke images of a single, all-revealing experiment or discovery. In fact it may be the case that we already have the data we need to arrive at an understanding of embryology, and the approach to such a subject lies in the direction of integration and rearrangement of a complex of existing concepts.

What might we imagine a "basic theory

of development" to be? Would it be something as conceptually simple as Mendelian genetics or as that all atoms are composed of a nucleus, mainly of protons plus neutrons, with set numbers of electrons whirling about them?

I believe that we must accept the fact that there can be no simple theory of development, any more than there can be a simple theory to embrace all of anatomy, or physiology, or behavior—unless we can be satisfied with "Development is the functioning of genes in embryonic cells."

And that, of course, is what embryology is. But embryologists are interested primarily in the products of gene action, not in the gene themselves. A variant of this basic concept applies to all life. So one answer to Horder's question "What is Life" is "Life consists of the activities of genes in organized cellular and subcellular systems." I suspect that those who ask that question hope for a different answer—possibly along the lines of Spemann's belief quoted before—that life is not only a manifestation of matter and energy but that there is something "more." Possibly there is but so far it eludes the most sophisticated methods and machines of science.

A Conceptual Basis for Developmental Biology

An attempt will now be made to construct *a* (not *the*) conceptual framework for developmental biology. It will be based largely on the hypotheses and data of those "grand masters of embryology," as Medawar referred to them, as synthesized by E. B. Wilson (1928, especially chapters 13 and 14), plus a modicum of updating based on subsequent observations and experiments.

A conceptual framework has value in relation to its ability to arrange data and ideas in a comprehensible and comprehensive system of thought. It is a way of looking at the natural world and finding associations among the diverse and seemingly unrelated phenomena. A conceptual framework for developmental biology will help students organize the various facts and ideas relating to embryonic development and to see developmental biology in its relations to biology as a whole. Needless to say there are many possible conceptual schemes for developmental biology. None will be fully correct nor fully complete—nor is such possible. A conceptual framework has value even when incomplete, or even partly inaccurate, since it provides a base to explore new puzzles.

The following numbered statements are of various types. Some are concepts, strictly speaking, others are not. Some are so well established that they are listed almost without comment, whereas others are expanded. The first two groups of statements relate embryos to life in general and to the history of life.

Concepts Relating to Cells

1. *Embryos are cellular.* They are living systems and so exhibit the basic properties of life, one of which is to be composed of cells. Hence, what cells can do embryos can do.

2. *Cells are complexly organized systems that consist of many interdependent and interacting parts.* Basic to all cell activities is the genetic code of DNA.

3. *The life and reproduction of cells is controlled by DNA.* Cells have the ability to replicate themselves and their DNA, events associated with mitotic cell division.

4. *The replication of DNA, nearly always precise, is subject to occasional error, or mutation.* The near constancy of DNA replication preserves the adaptations that have been built into the genome over the ages. The existence of rare errors is the basis of new adaptive possibilities.

Concepts Relating to Evolutionary History

5. *The origin of new adaptations is a consequence of natural selection acting on inherited variations.* This is the principal mechanism of evolutionary change.

6. *Evolution has favored those organisms with mechanisms for reproduction.* Theoretically individual organisms might be immortal but the forces of evolution have ignored that option.

7. *Reproduction involves the transfer of a cellular portion of the parent's body to a new individual(s).* Thus genetic continuity is always associated with reproduction.

8. *Reproduction is under Malthusian control.* Every species has the theoretical possibility of increasing to a population of infinite size. The resources required for life, however, are not infinite. Nevertheless there is a tendency for each species to increase to the limits of the carrying capacity of its environment.

9. *This tension between the increase in population size vs. an environment with finite resources puts a selective advantage on those individuals that can exploit new environments or exploit old environments in new ways.* This is the origin of the diversity of life—of today as well as of the past.

10. *The evolution of multicellularity has been one of the most successful adaptations.* Since most organisms of more than microscopic dimensions are multicellular, it is reasonable to conclude that this is the only effective solution that evolution has been able to devise for large and complex organisms. Increased size and complexity are associated with new ways of obtaining the resources required for life. With many cells there is the opportunity for groups of them to become specialized for different functions—that specialization making them more efficient in performing some function essential for the whole organism. But specialization means the loss of the ability to perform all of the functions required for the life of individual cells and the need for the cells with one type of specialization to depend on those cells with other types of specialization. In large, complex, cellular organisms the function of individual cells is for the welfare of the individual as a whole, just as the function of the individual must be for the benefit of the cells themselves.

11. *The evolution of large, complex organisms consisting of numerous highly differentiated types of cells requires new patterns of reproduction.* In comparison, single-celled organisms reproduce by dividing in half, the chromosomes undergoing mitosis. The daughter cells then grow to full size. Hence in single-celled organisms cell division and their reproduction is the same—and the requirement for genetic continuity is met.

This is not possible for multicellular organisms. In order to reproduce they cannot just split in half like an amoeba. They have evolved two basic ways of transferring parts of their body to their offspring—asexual reproduction and sexual reproduction.

12. *The forces of evolution have put a premium on sexual reproduction.* This involves the development of special cells, the gametes, in which the parental genes are segregated and independently assorted in an almost infinite number of combinations. At the same time meiosis halves the number of chromosomes that carry the genes. The union of eggs and sperm, when derived from different individuals, results in a still greater variety of genetic types. Some of these new genetic types of individuals might be able to invade new environments or be better competitors in their old environment. If so, their numbers would increase.

13. *Asexual reproduction, that is without the fusion of gametes, involves the formation of a new individual on the parent body, followed by the detachment of the new individual.* In some species, fragmentation of the body is followed by the regeneration of each fragment to form a whole individual. In both cases rigorous genetic continuity results, since the cells of the offspring are identical with those of the parent. Species that reproduce solely by such methods are regarded as evolutionary "dead-ends," since there is no possibility of genetic recombination.

Concepts Relating to Development

14. *Since sexual reproduction by multicellular organisms results in the formation of a single-celled zygote, complex mechanisms for converting that zygote into the multicellular adult with its diversity of differentiated cell types are required.* Thus development is required. The principal events in development are an *increase in cell number,* the *rearrangement of cells,* and, finally their *differentiation* and association as tissues and organs.

15. *Mitotic cell division is the universal way that individuals increase the number of their cells.*

(Since this discovery was made so long ago, in the 1850s, it has become completely incorporated in our thought patterns. Had the discovery been made in 1900, it would have been recognized as the embryological equivalent of Mendel's Law of Segregation—his First Law—and there would be fewer criticisms of embryology's lack of theory.)

16. *The embryo's cells become rearranged, usually drastically, and in their final positions become the primordia of the future structures.* There is essentially no variation in these cell movements in different embryos of the same species.

(Had these regularities been discovered in 1900, they would have been known as the Second Law of Development and note would have been made of the fact that this Second Law, plus the First, are as broadly applicable as Mendel's two Laws.)

CONCEPTS RELATING TO DIFFERENTIATION

Differentiation is the prime problem of developmental biology and a consideration of it will conclude this essay. Suggestions for a conceptual framework for this aspect of development will be based on these four axioms.

A. Cells are the biological units of structure and function.

B. Genes control the cellular syntheses and through them cell structure and function.

C. The gene-controlled cytoplasm can exert feedback control over gene activity.

D. Individual organisms are integrated systems that have overall control of their separate parts.

Axioms A and B are restatements of concepts 1–4 and are so well established that no more need be said about them.

Axioms C and D require explanation so far as their relations to ontogeny are concerned and they will be divided into several numbered concepts.

17. *The mature ovum is highly structured and has localized cortical and cytoplasmic determinants that largely control early development.*

Care must be taken here not to regard genes and cytoplasm as separate and antagonistic entities. They are two aspects of a functioning whole and totally dependent on one another. There is, however, a biological chain of command. The specificity of cells, organs, individuals, and species depends ultimately on the information encoded in their DNA. But the products of gene action—cellular substances and activities—may have feed-back control of the genes themselves. This cytoplasmic control is of enormous importance in early development.

Evidence for the importance of the cytoplasm accumulated in the late 19th century. A case that greatly influenced contemporary thought was Boveri's discovery that the chromosomes of the nematode, *Ascaris*, destined for the cells of the somatic tissues differ greatly from those destined for the gametes. Those of the germ line retain their form whereas those that will be in somatic cells fragment into many tiny chromosomes and, in fact, parts of the original chromosomes are eliminated (chromosomal diminution):

> By an ingenious study of centrifuged and double-fertilized eggs Boveri was able to establish the fact that the process of diminution is not an autonomous act on the part of the chromosomes but is induced by their cytoplasmic surroundings in the egg, a conclusion of fundamental importance for our general conceptions of development (E. B. Wilson, 1928, pp. 323–328).

A recognizably different cytoplasm, called the pole plasm, is present in a specific portion of the eggs of some insects. Nuclei that enter this zone are incorporated into cells that become gametes. If nuclei are prevented from entering the pole plasm, the embryos develop into adults that produce no gametes. It is possible to manipulate the nuclei to establish the fact that any nucleus forced to enter the pole plasm will become part of a gamete (E. B. Wilson, 1928, pp. 320–322).

Numerous examples are now known of the effects of the cytoplasm on the nucleus but, for our purposes, a dramatic example provided by Gurdon and Brown (1965) for the frog *Xenopus* will suffice. They studied the production of ribosomal RNA in early development. Essentially none is produced before gastrulation but thereafter the rate of synthesis increases rapidly. One can interpret this to mean that the rRNA genes are "turned off" before gastrulation and "turned on" thereafter. Using the techniques of nuclear transfer they removed an rRNA synthesizing nucleus from a neurula and injected it into an enucleated uncleaved ovum. Development began and the question was "Will the nucleus continue to synthesize rRNA or will its genes be turned off and synthesize none?"

One group of experimental embryos was allowed to develop to blastulae and then the amount of their rRNA measured. None had been synthesized. Another group of embryos was allowed to develop to the neurula stage and then their rRNA production measured. These had resumed rRNA production.

Thus the neurula nuclei had, in the cytoplasm of an early embryo, behaved as a nucleus in a normal early embryo. Then at the normal time—the cytoplasm's normal time—rRNA production began. We could say that those turned-on genes of the neurula were turned off by the cytoplasm of the early embryo and turned-on again at the normal time (see also Gurdon and Woodland, 1968, for more examples).

In mature ova and early embryonic cells the molecules responsible for the basic organization are situated mainly in the cortex. When we recall that early development is striking in the constancy of its events, it is not surprising that organization is built into the relatively stable cortex compared with the more fluid cytoplasm.

There is evidence of some organization in the more fluid cytoplasm as well. Wilson's observations on the determinants of the apical tuft of *Dentalium* suggest most strongly that the determinants were located first near the vegetal pole and then, in a few cleavages, were localized in cells near the animal pole. It would be hard for such shifts to occur in the cortex.

Most of the data, however, indicate that the determinants are to be found in the cortex. Those strikingly different pigmented areas of the cortex of *Crepidula* and other ova are so closely associated with the formation of specific embryonic structures that one suspects that they are at least markers, and may be the determinants in some cases.

The surprising results obtained by centrifuging eggs pointed to the importance of the cortex. Fertilized eggs could be centrifuged until the cytoplasm was divided into layers of materials differing in density—all the yolk granules at the bottom and all the oil drops at the top, for example. Nevertheless such embryos developed normally or almost so. However, when greater centrifugal force was used, enough to disrupt the pattern of the cortex, then abnormalities were observed.

The importance of the cortex was emphasized by Just (1939) and dramatically demonstrated by Curtis (1962), who showed that the cortex of the gray crescent of the amphibian, *Xenopus*, could be transplanted and induce a secondary embryo. Thus the determinants, or the primary organizer, that Spemann and Mangold found in the dorsal lip are already present well before the onset of gastrulation (see also Pasteels, 1964).

Cytoplasmic localization was well known to the grand masters of embryology but this was not always understood by others. After 1900 the rapid rise of genetics, compared with the measured tread of embryology, left many biologists with the opinion that cells, especially those of embryos, were somewhat leaky bags of assorted molecules awaiting instructions from the genes. New discoveries found the genes doing more and more things and soon nothing seemed to be left for the cytoplasm. Thus what was clear to E. B. Wilson and others by the early 1890s ceased to be part of a general theory of development.

Part of the problem was the apparent simplicity of ova. There is no question but that many mature ova appear to be "simple cells." Apart from a few mosaic eggs, with their visibly differentiated cortex, ova seem to consist of a cell membrane, a nucleus, assorted granules, oil droplets, and yolk granules, all uniformly distributed throughout the cell. There was little to compare with the complexity of the highly differentiated cells of adult organs and tissues. Many protozoans appeared to be more complex than ova. Since these ova looked simple, researchers felt that they must be simple.

Simple ova made for complex problems so far as a hypothesis for differentiation was concerned. What mechanisms could convert a simple, undifferentiated, homogeneous, generalized egg into an embryo? It was hard to see how novelty could arise from such a beginning (our old problem with epigenesis again). One could imagine mitotic cell division dividing that simple cell into a ball of identical simple cells. Once there was a ball—a solid blastula, perhaps—there would be the possibility of an external stimulus. Some of the cells would be on the outside and others on the inside. One might suspect that those two regions would be stimulated in different ways. The outer cells would have a better oxygen supply and the possibility of eliminating carbon dioxide and other wastes more readily. Finally, if that ball of cells dropped to the ocean floor and stuck, there would be a top and bottom. One could imagine the formation of an individual with a structure similar to Haeckel's *Olynthus.* Our organism would be radially symmetrical, differing in latitude but not longitude.

18. *The organization of the ovum is largely determined by stimuli from without.* It appears that the mature ova of all animal species are organized to a considerable degree by the time of ovulation. This basic organization is established under the influence of maternal genes. While the ova are in the ovary they are not isolated individuals. They are cells of the mother's body, formed from preexisting maternal cells, and supplied with the requisites for life. Since ova are cells of the adult female we should find it no more of a problem to accept that they are organized than to accept that the mother's neurons, kidney cells, or cells of the Islets of Langerhans are highly organized.

If we accept that the ovum has already taken many steps along the route of becoming a new individual during oogenesis, a difficult technical problem emerges for the developmental biologist—ovarian eggs cannot be manipulated with the same ease as early embryos of amphibians, sea urchins, or ctenophores. Hence clues had to be sought through correlations between the organization of the oocyte and external conditions. Many were found. For example, in many species of marine invertebrates the basic polarity—the animal pole–vegetal pole axis—is determined by the position of the egg in the ovary. Another example comes from insects, many of which have elongate ova and the long axis of these often parallels the main axis of the adult body.

The egg that seems to have the least organization at the time of ovulation is that of the marine alga, *Fucus* (D. M. Whittaker, 1940). Almost immediately, however, a protuberance appears on the undivided egg and at first cleavage the egg divides into two unequal cells (Fig. 58). The fate of these two cells is established at this time. The larger cell becomes the thallus and the one with the protuberance becomes the rhizoid. So far as Whittaker could tell, the formation of the protuberance, which sets future development, is due to some external influence. He suspected this when he noticed that in groups of cells the protuberance formed inward, as shown in Figure 58. This suggested that maybe the concentration of some substance produced by the cells was the stimulus. In addition, tests of various environmental components, such as pH, light, and temperature, showed that the site of the protuberance could be manipulated at will (Fig. 58).

By experimental means, therefore, a fundamental step in differentiation could be controlled. Genes that happen to be allocated to the cell with the protuberance will become active in the formation of the rhizoid. Those entering the other cell at the first mitotic division will participate in the formation of the thallus. It would appear, therefore, that what genes do can be affected by external factors working through the cytoplasm in which they function.

Another example of external stimuli affecting the organization of the early embryo relates to the origin of bilaterality. This is the reason why those observations of Newport, Roux, and others on the

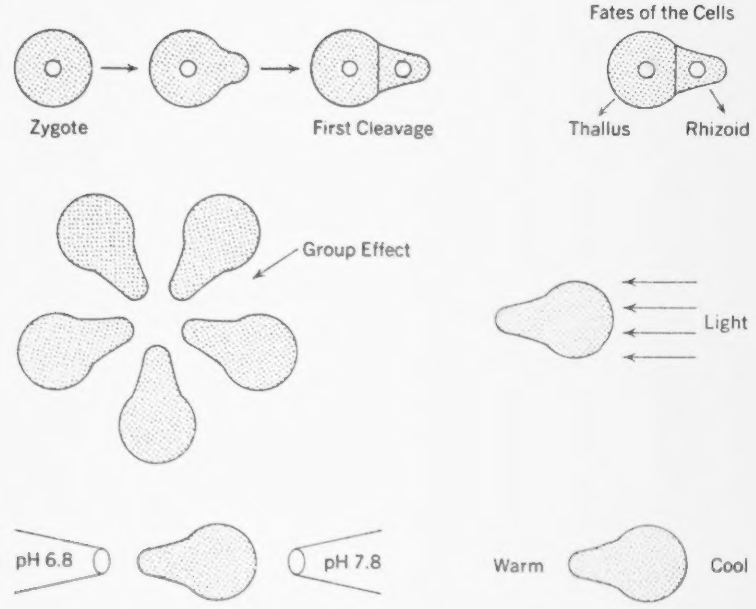

FIG. 58. The early development of *Fucus* and some of the factors that influence the formation of the protuberance.

entrance point of the sperm were so electrifying. Here was a stimulus that not only determined the position of a gray crescent but also marked the plane of first cleavage, the position where the dorsal lip was to appear, and finally the anterior–posterior axis of the embryo and adult.

19. *Each cell receives a complete set of genes and different ones of these are expressed in different ways in different embryonic cells, controlled in part by specific cytoplasmic molecules of both cortical and non-cortical regions.*

The Roux-Weismann hypothesis of qualitative nuclear division was short lived and most of the grand masters came to accept the hypothesis that all cells receive the same set of genes—and what the cells did with them in very early development depended largely on the cytoplasm. This hypothesis was hard to prove because one could not at that time study the genetics of somatic cells.

Nevertheless the indirect evidence was fairly good. The data on regeneration of planarians and hydroids seemed to indicate that all cells retained the full genetic capability of the species.

The isolation of blastomeres of regulative eggs showed that a full set of genes goes to each cell, at least for the first few cleavages. When differences among the chromosomes of somatic cells was recognized, it became possible to trace them throughout successive mitotic divisions and find that all somatic cells have the same set of chromosomes. This individuality of the chromosomes (III, pp. 653–657) was evidence that all cells are genetically equivalent.

None of these earlier investigations was wholly convincing and it was not until Briggs and King (1952) and later Gurdon (1962) perfected methods for transferring nuclei from older embryos and differentiated cells that better data were available. The technique is shown in Figure 59. Cells from a blastula, or a later embryo, or even an adult can be dissociated and then injected into an enucleated ovum. Normal development occurs in varying percentages of the cases, depending on the source of the nuclei. In some cases sexually mature adults have been obtained from these fatherless embryos.

That even some nuclei from differen-

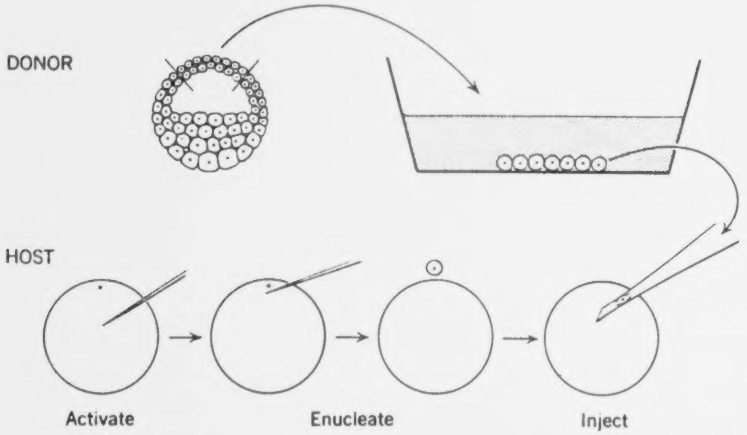

FIG. 59. The Briggs and King method of transferring nuclei. A piece of an older donor embryo, in this case the roof of the blastocoel, is removed and placed in a solution that causes the cells to disassociate. A donor egg, fresh from the uterus, is then activated by a jab with a glass needle and its nucleus removed by flicking it out with a glass needle. A single cell donor is drawn into a pipette and then injected into the host embryo. No sperm are involved and the host develops with the injected donor nucleus.

tiated cells have the ability to support normal development is taken as evidence that any nucleus can do so.

However, one cannot conclude from such results that all somatic nuclei are undifferentiated. One can only conclude that they are not irreversibly differentiated. It is beyond argument that the nuclei of differentiated cells are differentiated. The fact that an erythrocyte of a frog synthesizes hemoglobin, but not pepsinogen and a stomach cell synthesizes pepsinogen, but not hemoglobin shows that different genes are active in the two cell types.

20. *Embryos are integrated systems with the whole having overall control of the parts.* There are innumerable examples of the whole embryo or adult organism controlling its parts and the mechanisms are varied. Holtfreter's experiments on the transplantation of pieces of early gastrula ectoderm to older embryos and finding that each developed according to its surroundings is a fine example (Fig. 56). Hörstadius's combinations of different cell layers in *Paracentrotus* find their explanation less in the specific layers involved than in the portions of the gradients they contain. Each blastomere of the two-cell stage of amphioxus or *Echinus* has the capacity to produce an entire embryo but that capacity is restrained when each is part of a whole embryo.

The fate of an embryonic cell reflects its position in the whole embryo rather than its innate capacity.

Some of the more dramatic examples of the control of the whole over its parts come from experiments on regeneration. Consider the case of a planarian flatworm cut in half—across the long axis of its body. Each half will undergo an extensive reorganization and produce a complete planarian. The events in regeneration can be explained by assuming the presence of a gradient (Child, 1941). The "high" point of the gradient is the head end and there is a gradual decline in its effect until we reach the tail.

When the body is cut crosswise the anterior half will regenerate a tail at its hind end. The posterior half will regenerate a head at its front end. The cells at the posterior end of the anterior half and the cells at the anterior end of the posterior half were adjacent before they cut so they should have been as similar to one another as is possible to imagine. Nevertheless their fates in regeneration are entirely different. The conclusion is inescapable that the newly regenerating whole is controlling what happens in its parts.

That last statement reflects a truly extraordinary biological phenomenon. Let us consider some of the implications. A planarian when cut begins to regenerate and stops when its body is complete. What stops this regeneration? Why does it not continue as a cancerous growth forever? Each fragment must have the complete information on "How to make a whole planarian" and also a mechanism to shut off regeneration when the complete body has been formed. In the case of planarians the marvel is not only that the lost part is restored but that each fragment is totally reformed. The entire structure is altered in each fragment so that at the end of regeneration a perfect, though small, planarian is the result. (There is no feeding or growth during this period.)

The Basic Pattern of Ontogeny

These 20 concepts can provide a framework to organize the data of developmental biology.

At the time of fertilization the ovum is a highly structured system with the determinants for the early stages of development arranged in a definite order, mainly in the egg cortex. The ovum was part of the adult female and its genetically determined organization was laid down during oogenesis.

This system is set in operation by the entrance of a monoploid sperm and its fusion with the monoploid egg nucleus.

The diploid zygote undergoes a series of cleavages that divide not only the original cytoplasm of the ovum but also the cortex into a number of cells. Mitosis gives each cell a full set of genes but they will come to occupy cells that differ in their specific cytoplasms and differ especially in their cortex.

These cytoplasmic localizations and cortical differences among the cells result in different genes being activated in different cells. This is the initial cause of cell differentiation.

Once the embryonic cells have begun to differentiate, the interactions among them will lead to further differentiations.

The position of cells, from the earliest stages, determine their fate—that is, the structures they will form. Every portion of the later embryo is to be found in a specific location in the embryos of earlier stages.

A rearrangement of cells occurs during gastrulation and at its close the cells are in groups that will then differentiate into the structures in an exact manner. This differentiation often involves the control of one group of cells by another, as in the amphibian organizers.

At the time of fertilization the eggs of some species have pronounced regional differences of cortex and cytoplasm. These are the mosaic eggs with their determined parts which, when isolated, will usually self-differentiate. By contrast, the cells of the regulative species are less determined. In any event even these eventually reach the mosaic stage.

Even in the most strictly mosaic species, however, the period of strict determination may be transitory. For example we find that annelids and mollusks, which have highly mosaic eggs, regain a high degree of regulative ability when they are adults. Then they can regenerate whole bodies from parts.

Thus we can account for differentiation by assuming that the genes of the zygote find themselves in an environment where different genes are activated by cortical and cytoplasmic molecules that were, themselves, produced and ordered under the influence of the maternal genes. Differentiation is not just a matter of genes and cortex and cytoplasm interacting with each other but also of the genes of one generation controlling those of the next generation during the period of very early development.

I believe that we do have a conceptual framework that will account for embryonic development. Some of the basic concepts cannot be rigorously defined—the control of the whole over the parts, for example—but there is no doubt whatsoever that such control exists.

Thus the grand masters have left us a framework that allows us to comprehend what has been discovered and that can serve as a basis for further analysis at the level of cell and organism. It can, as well, serve to extend the analysis to the molecular level.

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AMER. ZOOL., 27:575–579 (1987)

Embryonic Induction[1,2,3]

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SYNOPSIS. "Spemann's" lecture treats experiments on the separation of the first two cells of a frog, or sea urchin, or salamander embryo; the induction of a lens in a frog embryo by an optic vesicle (primordium of the eye); and the primary organizer that is a dynamic center, establishing the basic organization of the embryo and inducing the nervous system and sense organs. "Spemann" goes beyond science in speaking poetically of the beauty and order in the universe, and to illustrate how a good scholar should work he uses a lovely metaphor of piecing together the fragments of a broken vase. "Spemann" concludes with a stirring plea for academic freedom.

Ich danke Ihnen Herr Professor Moore. Meine Damen und Herren. It is great joy to begin my lecture with ladies and gentlemen instead of by *Heil Hitler*, as we must say in my country these days. I am sorry I do not speak English very well, although I studied your language already beginning in the *Gymnasium* in Stuttgart, my city of birth. "Do you know where Stuttgart lies?" asks an old German verse taught to me by a nursery maid. "*Wisst ihr auch, wo Stuttgart liegt? Stuttgart liegt im Tale, wo's so schöne Mädle gibt, aber so brutale.*" In translating "*brutale*," I suggest that you do not say: beautiful maidens of Stuttgart are brutal. You should say: beautiful maidens of Stuttgart are devastating. I know from experience. I read English easily. My favorite American authors are Whitman, Emerson and Thoreau. I have travelled a little in England and America. I gave the Croonian Lecture before the Royal Society in London in 1927 and the Silliman Lectures at Yale University in 1933. The Silliman Lectures were the basis of this book on embryonic development. [Spemann picks up the German edition of the Silliman Lectures.] In it I have summarized many of the experiments performed by my students, associates, and me. Only three studies will be discussed in this lecture. Incidentally, in the preparation of the English edition of my book I had the assistance of a young man from the University of California, Berkeley: Richard Eakin.

The first study is on the separation of the first two cells of a salamander embryo. This was done at the University of Würzburg, where I took my doctor's degree under the great Theodor Boveri, who had a profound influence on me. Boveri was not only a fine scientist but also a very kind man. I remember especially his solicitude at the time of my doctor's examination, a very formal occasion in the German universities of that time. After finishing one's thesis a doctoral candidate must appear before his examiners in full dress with tails, striped trousers, white gloves, and—and—*Wie heisst Zylinderhut auf English?* [Help comes from Professor Moore.] Top hat? *Jawohl.* Top hat. Very colorful word. So. The candidate arrives at ten o'clock in the morning and awaits alone until his examiners assemble and finish their conversation. It was then that Boveri came to me with words of comfort. After two hours the examination is broken for lunch, which is served to the professors at the expense of the rektor of the university. All doctoral examinations are open to any professor, but most of them come only for the free sandwiches and wine. Meanwhile, the unhappy candidate does not dare to touch the wine, because the rest of the examination is yet to come. If the candidate passes, the next morning he must put on

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[1] From the Symposium on *Science as a Way of Knowing—Developmental Biology* presented at the Annual Meeting of the American Society of Zoologists, 27–30 December 1986, at Nashville, Tennessee.

[2] Originally published in *Great Scientists Speak Again*, by Richard M. Eakin, copyright (©) 1975 by The Regents of the University of California.

[3] Illustrations for Spemann's lecture may be found in John Moore's essay for this symposium.

[4] An impersonation by Richard M. Eakin, Department of Zoology, University of California, Berkeley, California 94720.

his formal clothes again and call upon each of his examiners at their homes.

At that time there was a controversy in biology between two experimentalists, Wilhelm Roux of Halle and Hans Driesch of Leipzig, regarding the basic character of development. Was the embryo a mosaic of predetermined parts, as claimed by Roux, or was the embryo indeterminate and regulative, the position of Driesch? Roux had worked with the two-celled embryo of a frog. He killed one of the cells by pricking it with a heated needle. The surviving cell formed only a half of an embryo. According to Roux this was because the nucleus of that cell had the determinants for only a half of the embryo. Driesch, on the other hand, obtained quite different results with an experiment on two-celled embryos of the sea urchin. By vigorous shaking of a tube of sea water containing embryos in the two-cell stage, he succeeded, in a few instances, in breaking the egg membrane which allowed the two cells to separate from each another. Each developed into a whole embryo. Who was correct? Roux or Driesch?

I took up the problem in Würzburg after my doctor's degree. I used a still different technique on the two-cell embryo of a salamander which, like the frog embryo, lies inside a membrane and several layers of jelly. I made a fine noose of baby's hair which I stole from the head of my first son. The noose was slipped over the jelly around a two-cell embryo and then slowly tightened, constricting the embryo until the two cells were separated. Each cell developed into a whole embryo. Thus, I had formed a pair of twins from one egg, in agreement with Driesch. Accordingly, both echinoderms and chordates are indeterminate and not mosaic in their development.

But it turns out that nature is not always so simple as one thinks or would like it to be, as I discovered in my noose experiments. Sometimes, I did not get twins. Why? I soon discovered the secret. There is a region on a fertilized amphibian egg—frog or salamander—called the gray crescent. This had been known for some time. It is a lightly pigmented, crescentic area which appears near the equator of the egg shortly after fertilization. Usually the first cleavage cuts the gray crescent so that each of the first two cells gets a part of it. But sometimes the first cell division divides the fertilized egg so that one cell receives all of the gray crescent and the other has none. I discovered that if the noose constricted an embryo in the first instance (sagittal cleavage) I obtained twins, but in the second type (frontal cleavage) there developed one normal embryo and a *Bauchstück—Wie heisst Bauchstück?* [Help comes again from the audience.] Yes, belly-piece. A belly-piece is made of skin and gut but it has no notochord, no neural tube, no eyes, no ears, *und so weiter.* Thus you see that the gray crescent is vital for the normal development of an embryo. I have often thought that I should write a love song entitled, "Without my gray crescent to embrace me I am just a belly-piece." [Spemann makes a large crescent with outstretched arms which close in a hug.]

Also. The amphibian embryo is not so completely regulative and indeterminate as suspected. It has a touch of mosaicism. The grey crescent is a special region. It must contain something essential for the development of notochord, nervous system and sense organs. I did not fully understand these results until several years later when I discovered the organizer, which I shall describe later.

Now, not only can a whole embryo develop from half of an egg, but a whole embryo can develop from two eggs fused together. Take two eggs after first cell division, remove the jelly and egg membrane from both, and place one on top of the other. They will fuse together, continue to divide, form a ball of cells which develops into a normal embryo of giant size. One can even fuse the embryos of two different species of salamanders and obtain a chimera, that is, an embryo which is normal in every respect but constructed from cells belonging to two different species, as shown by two of my colleagues. The power of nature to heal and to regulate and to overcome adversity is fantastic.

I now discuss a second work—on the development of the vertebrate eye. These experiments were also conducted at Würzburg when I was a *Privatdozent,* which position corresponds to your lecturer. The *Privatdozent* in the old German system, however, did not receive a salary. He collected fees from the students who attended his lectures. The professor, only one in each field in a university, always lectured in the large general courses, so he collected the largest number of fees, in addition to his salary. A *Privatdozent* lectured to small classes and consequently received few fees. Indeed, he must have another source of revenue or starve.

For transplantation of small parts of amphibian embryos special techniques were needed. I used very simple instruments: glass-needle knives to cut the soft embryos, a loop of baby's hair mounted in a glass capillary tube to manipulate an embryo, balled glass rods to make depressions in wax to cradle an embryo, and tiny glass bridges to hold grafts in position after they have been transplanted. I never ceased to marvel at the beauty of an embryo, how tender yet how swift to heal and repair any damage inflicted upon it. For hours I could sit and watch an egg cleave, or the cells move through the blastopore, or the neural folds rise and fuse to form a neural tube, or the tailbud embryo move in circles within its membrane by the beat of thousands of cilia. [Spemann becomes mystical.] Some scientists see the beauty and order in the structure of the atom, or in the sweep of the Milky Way and the swing of the Pleiades, or in the homeostasis of blood sugar, or in the adaptations of birds for flight, or in the color and patterns of insects; but I found them in the embryo.

And what a glorious society we would have if men and women would regulate their affairs as do the millions of cells in the developing embryo. We should study nature for moral lessons. This injunction has been better stated many times before. Bryant wrote in "Thanotopsis": "Go forth under the open skies and listen to the teachings of nature. She speaks a various language." *Auf Deutsch, en français,* in English. And Shakespeare said: there are "tongues in trees, books in running brooks, and sermons in stones." And in the Bible we read: "Go to the ant, thou sluggard. Consider her ways and be wise." [Spemann realizes that he has drifted away from his subject.] But now we go to the embryo.

I learned to remove and to transplant the embryonic eye—called the optic vesicle—which forms as an outpocketing, like a balloon, from each side of the embryonic brain. When this optic vesicle grows to contact the skin ectoderm, the outer covering of an embryo, it invaginates to form a cup, called the optic cup. The inner thick layer of the cup becomes the retina. The lens of the eye, however, arises not from the outgrowth of the brain but from the skin ectoderm.

We are now ready for some experiments. Remove an optic vesicle at an early stage in its development in a frog embryo by cutting the optic stalk—so. Result: not only no eye, but also no lens. Without the stimulus provided by the optic vesicle, the skin ectoderm will not form a lens. To be sure of my conclusion, I conducted another experiment. I transplanted a young optic vesicle beneath some *belly* ectoderm. A beautiful lens was there formed by the ectoderm. Normally, of course, lenses do not grow on bellies. [Spemann chuckles.] But given the stimulus, whatever it is, from an optic vesicle, skin ectoderm of a frog embryo responds with the proliferation of a mound of cells that transforms into a crystalline lens. Some qualifications about age and species of frog embryo used might be added, but they would not alter the basic principle I have just illustrated. I called this principle *induction.*

As you may be acquainted with my work on the dorsal lip organizer, I shall be brief about my third topic. By dorsal lip I refer, of course, to the upper lip of the blastopore, the opening in the early embryo through which cells move to form future internal organs. Those cells rolling in dorsally give rise to skeleton and muscle. More importantly, however, they determine the destiny of the overlying external cells, inducing them to become brain, spinal cord, eyes, and ears. So decisive is its inductive action that I termed this dynamic center of the embryo the primary organizer. It was this work which won the Nobel Prize in Medicine and Physiology in 1935, although the crucial experiment was done much earlier, published in 1924, in collaboration with my graduate student and research assistant, Hilde Mangold. In that experiment she transplanted the dorsal lip from an early embryo (donor) of one species of salamander into the side of another embryo (host) of the same age but of a different species. By cross-transplanting between two species, which differ in size of cells and amount of natural pigment, it was possible to distinguish between donor and host cells at a later stage of development. The transplant organized (induced) a new embryonic center on the side of the host. The former I called a secondary embryo, the latter the primary embryo. In rare instances, a secondary embryo may develop into an almost perfect individual attached to the side of the host. Now it became clear why the gray crescent region of the fertilized egg is so important for development. Without it, you remember, only a belly-piece forms. The reason was now clear: the gray crescent marks the future dorsal lip area; hence, it is the forerunner of the organizer.

In the last chapter of my book I discuss some general topics including my ideas on how the good scientist should work. I have found an analogy to an archeologist very helpful. Let me read a paragraph to you as it will appear in the English edition of my book. [He removes a card from the German edition and puts on a pince-nez.]

I should like to work like the archeologist who pieces together the fragments of a lovely thing which are alone left to him. As he proceeds, fragment by fragment, he is guided by the conviction that these fragments are parts of a whole which, however, he does not yet know. He must be enough of an artist to recreate, as it were, the work of the master, but he dare not build according to his own ideas. Above all, he must keep holy the broken edges of the fragments; in that way only may he hope to fit new fragments into their proper place and thus ultimately achieve a true restoration of the master's creation. There may be other ways of proceeding, but this is the one I have chosen for myself.

Perhaps I can make my point better with a demonstration. I have here two inexpensive vases which I bought in one of your 5- and 10-cent stores. Incidentally, you should call them dollar stores. This one is unbroken. But this one has been smashed. Perhaps kitty knocked it off my desk. [Spemann scatters the pieces on the podium.] Suppose—just suppose—that these are very valuable vases or that they hold dear memories for me. I should like to restore the broken one. So I take the pieces to an artisan. And I tell him: My good fellow, please put my vase together. Restore the thing of beauty. To be faithful to my analogy with the archeologist, I must not give the artisan the unbroken vase. [Spemann places the whole vase to one side.] He has only a handful of shattered pieces. He does not know what the whole vase is like. And so he begins by trial and error to fit the pieces together. [Spemann picks up two pieces of the broken vase.] Now here is the critical point. He must not work according to his own ideas. That is, he must not force pieces together. He must keep holy—*heilig*—the edges of the fragments. If the pieces match, they will fit together without alteration and without pressure.

And so the good scientist works, with fragments of truth, piecing them together carefully and critically, to bring forth the whole truth, the thing of beauty. "Beauty is truth, truth beauty," said Keats. As he works he must have no convictions of his own—hypotheses yes, but no conclusions. No preconceived ideas, no prejudices, and no allegiances which will impair the objectivity of his research. This is what I mean by inner academic freedom. When I sit and reflect upon beauty and harmony in an embryo I am Hans Spemann the philosopher, I am not Hans Spemann the scientist. In the white heat of research or in the critical evaluation of the work of another scientist matters of religion, politics, social position and personal gain are to be rigorously excluded.

Now I go one step further. Not only must the scientist have academic freedom within himself, but he must be free from all outside pressures as, for example, those from government. In my country at the present time *Akademische Freiheit* is being destroyed. It is my sincere wish that you, students and professors, will never be subjected to this kind of tyranny. And so, ladies and gentlemen, it has been a pleasure to address you. *Danke schön und aufwiedersehn.*

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Understanding Embryonic Development: A Contemporary View[1]

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SYNOPSIS. The immediate causal explanation for cell differentiation is the regulated ontogenic expression of specific sets of genes in cells of each given type. Demonstration of this principle has resulted in a revolutionary reorientation of the study of early development. Imposition of spatial patterns of differential gene expression at the onset of *Drosophila* and sea urchin development is briefly discussed. The mechanisms by which this process occurs in these two systems differ in fundamental respects. Molecular analysis of differential gene function in the embryo promises to provide a fruitful approach to classically defined questions, such as the nature of induction, regulative development, and cytoplasmic localization.

CHANGING IDEOLOGICAL ORIENTATIONS IN THE STUDY OF EARLY DEVELOPMENT

The great problems of embryonic development that we now recognize were largely defined by the close of the classical period of thought and discovery in this field, about a half century ago. The reality of *epigenesis,* the *de novo* development of complex biological forms from the structurally indifferent egg, had been established through the painstaking studies of 18th and early 19th century observers, following post-renaissance antecedents, and even earlier insights, extending back to Aristotle's *De Generatione Animalium* (see the essay of J. A. Moore (1987) in this volume; Meyer, 1939; Needham, 1959). In the late 19th and early 20th century the epigenetic phenomenon of embryonic development was dissected, ramified, subdivided, and recast: various embryos were found to be constituted of precise cell lineages; maternal determinants of morphogenetic significance were localized in specific regions of egg cytoplasm, before or after fertilization; specification of embryonic structures was shown to be due in some cases to induction; and certain embryos were shown to be able to regulatively reproduce missing parts. From a modern perspective the most prescient and significant achievement of the classical period of cell and developmental biology was the theory of cellular heredity, which placed the hereditary determinants of the organism in the nucleus of each and every cell, and which leads directly to our present definition of cell differentiation. The demonstration that developmental processes are ultimately controlled by, and require, the activity of the genome was of fundamental importance (for reviews see Wilson, 1896, 1925; Davidson, 1986*a*, *b*). It remains the task of modern developmental biologists to interpret the major phenomena of embryogenesis that were identified and discussed by our predecessors. Nonetheless, in the last 20 years, the basic conceptual orientation of this field has undergone a most important and striking shift.

The least exclusive and most general characterization of this shift may be that the central thrust of effort in developmental biology has turned away from phenomenology, however elegant, and toward mechanistic explanation at the cellular level. While this may appear a simplistic platitude, the effect has been profound, in respect to the nature of the explanations proffered, and the subjects chosen for research. In some areas the result has been a significant narrowing of focus, at least until very recently. For example the study of gross Spemannian inductive processes, so long a dominant activity in developmental biology, has in

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recent decades greatly receded in relative importance, because this phenomenon has proved so refractory to mechanistic explanation at the cellular level. So, for the same reasons, have other problems on which classical interest was once brilliantly focussed, such as localization and regulative development. On the other hand the problem of cell differentiation in the early embryo has moved to the scientific forefront, and now is under intense investigation in several different biological systems. It is in this particular area that it is most likely that satisfying explanations will soon emerge, thus providing the molecular mechanisms underlying the initial patterns of differentiation observed in these embryonic systems.

The advent of new paradigms from other areas of cell biology, new technology, and above all, new theoretical orientations, have all contributed to the realignment of the conceptual axis of developmental biology. In my opinion the most important and fundamental ideological intrusion has followed from the general molecular level confirmation of the *variable gene activity theory of cell differentiation.* This theory states that different specific sets of genes are expressed in given differentiated cell types, though in general all the cells retain the total genome of the organism, and that the expression of the cell type-specific sets of genes constitutes an immediate and sufficient explanation for the particular biochemical, structural, and functional specializations displayed by these cell types. There are logically consistent alternatives, all of which have in the past been proposed, and some of which indeed find legitimate application in certain unusual biological systems or cell types. Among these alternatives are the possibilities that the relevant specific properties of cells are not directly controlled by their nuclear genes, but are rather cytoplasmic functions of their environments; that cells of each type contain different fractions of usable genome, rather than the complete genome, so that the significant regulatory event generally required for differentiation is a cell type-specific reorganization of the genome; that all genes are expressed all the time in all cells, but with variable "penetrance", etc. The idea of variable gene activity is of course not recent in origin, having been foreshadowed by many classical observations (see review of this discussion in Davidson, 1986*b*). The first explicit enunciation of this theory, to my knowledge, occurs in a passage written by T. H. Morgan in 1934, towards the end of the classical period of developmental biology:

> The implication in most genetic interpretations is that all the genes are acting all the time in the same way . . . . An alternative view would be . . . *that different batteries of genes come into action as development proceeds* . . . . The idea that different sets of genes come into action at different times . . . [requires that] some reason be given for the time relation of their unfolding. The following suggestion may meet the objections. It is known that the protoplasm of different parts of the egg is somewhat different . . . and the initial differences may be supposed to affect the activity of the genes. The genes will then in turn affect the protoplasm . . . . In this way we can picture the gradual elaboration and differentiation of the various regions of the embryo.

The complete and convincing *demonstration* of this concept had to await the identification of DNA as the genetic material; of messenger RNA; of cell-specific protein synthesis; and of transcriptional processes. Certainly one of the major achievements of biology between 1955 and 1980 was the overwhelming accumulation of evidence that *in general* terminally differentiated animal cells of given type do express different sets of genes, and that differential, ontogenic regulation of gene activity indeed provides the immediate causal explanation of cell type specificity.

"REGULATORY ARCHITECTURE" OF THE EMBRYO: HOW ARE DIFFERENTIAL PATTERNS OF GENE ACTIVITY INSTITUTED?

Couched in contemporary terms, the heart of the ancient puzzle of epigenetic development lies in the mechanisms by which differential gene activity is established in various regions of the embryo. Though the issue is singular, the answers are likely not to be. Thus the kinds of regulatory functions required in embryos that utilize diverse modes of development are probably quite different from one another. Some of the basic distinctions amongst pathways of embryogenesis that *a priori* would seem most significant for the implied regulatory architectures are: the extent of reliance on determinant cell lineages that segregate from one another early in cleavage; the significance and timing of early intercellular interactions, and of cell migration, if any; the degree of spatial organization in the egg at fertilization; and the timing of development and the approximate number of cells in the embryo at the onset of regional cytodifferentiation.

A few examples may illustrate the deep distinctions amongst embryos of various groups in respect to these properties. The mode of development described for the nematode *Caenorhabditis elegans* represents one extreme. The complete cell lineage of this embryo is known (Sulston *et al.*, 1983), and except for a very small number of the 550 or so cells of which the first stage larva is composed, the fate and specificity of every cell can be predicted from its position in the invariant lineage map. Some lineage elements give rise to clones of cells that all perform the same function, but in most of the lineage, programmed asymmetric divisions produce cells of diverse type. The founder cells giving rise to each unique lineage element inherit specific sectors of egg cytoplasm, and thus it is reasonable to assume that the fates of the progeny of these founder cells are specified initially by regulatory factors of maternal origin, that by the onset of cleavage are regionally localized in the egg cytoplasm. Ablation and other experiments carried out on *C. elegans* embryos confirm that the founder cell lineages develop in an autonomous fashion, and that at least in certain cases factors conferring lineage-specificity are resident in the founder cell cytoplasm (*cf.* Wood *et al.*, 1983, 1984). Except for a few cells inductive interactions are not important in this embryo. Several very interesting genes have been discovered, mutations in which alter the developmental pattern of certain cell lineage elements, and the consequent cell specifications, though it might be noted that so far most such mutations affect postembryonic developmental processes (reviewed by Sternberg and Horvitz, 1984). It is clear from these examples, and from lineage comparisons with related species (Sternberg and Horvitz, 1982) that there are developmentally expressed genes which precisely and autonomously regulate the fates and behavior of specific lineage elements in *C. elegans*. The mode of early development in higher vertebrates contrasts with that of *C. elegans* in almost every respect. There are no fixed early cell lineages in teleost, amphibian, avian, or mammalian embryos. In these embryos the progeny of a majority of individual blastomeres include cell types of many kinds, even if the test blastomeres are selected from advanced pregastrular stages, and in no two embryos is the postgastrular distribution of these progeny likely to be identical. There is very extensive cell migration, and many of the cell specifications depend on inductive interactions by which the subsequent fates of large assemblages of cells that occupy given positions are regionally determined (reviewed by Davidson, 1986*b*). Cytodifferentiation in these embryos occurs only when there are many thousands, rather than only a few hundred cells. Autonomously differentiating, determinate cell lineages eventually appear, but only late in development, *e.g.*, the hematopoietic lineages that continue to differentiate in the bone marrow in postembryonic mammals. Maternal cytoplasmic determinants are required to set up the global spatial coordinates of the amphibian embryo, some of which are localized by cytoplasmic movements following fertilization (reviewed by Gerhart, 1987), but there is no evidence at all for such determinants in mammalian eggs. Certain particular forms of *regulatory* requirement are implied by the biological processes of higher vertebrate embryogenesis. Thus, for example, intercellular trigger-receptor systems linked to gene regulation networks are suggested by the widespread inductive mechanisms. On the other hand, genetic mechanisms that operate by specifying cell fate as a parameter of cell lineage can play little role in the vertebrate embryo until after its regional diversification has already been established. In general, in this mode of embryogenesis, most cells remain pluripotential and plastic throughout a large number of divisions, and the batteries of cell type-specific genes ultimately selected for activation in given cells depends on their eventual position in the embryo. It would thus seem most unlikely that we could learn much about the structure of the gene regulatory networks that control the initial spatial organization of differentiated cells in *C. elegans* development by studying those that control *Xenopus* development, or *vice versa*, although ultimately, precise cell lineages occur in the amphibian larva, just as induction is ultimately required in the *C. elegans* larva.

Metazoan developmental regulatory systems are almost certainly hierarchial. The functional properties of each cell type, including the specific proteins it contains, its shape, motility, and role, are generated by the batteries of structural genes that it expresses. These can be conceived as the lower level of the hierarchy, and the regulatory genes that control them, in pleiotropic fashion, as constituting the upper level (see Britten and Davidson, 1969, 1971 for discussion of logical aspects). Knowledge of the processes by which differential patterns of gene expression are initially established is at present most advanced for *Drosophila* and for sea urchin embryos. The modes of dipteran and echinoid development are again extremely different, and in addition the kinds of information that have accrued are dissimilar, due to the peculiar experimental opportunities offered by each system. *Drosophila* developmental biologists have succeeded in characterizing a number of important regulatory genes that may belong to the upper levels of the developmental hierarchy. These genes have been identified esssentially by searching for mutations that catastrophically and often pleiotropically affect body form. In modern times many of these putative regulatory genes have been successfully characterized at the molecular level. However, though in some cases we now know their structure, the spatial distribution in the embryo of their immediate products, and the effects on the developmental process of deleting their functions, we yet understand very little of what structural genes they actually regulate. To obtain a global knowledge of the *regulatory systems* of which such genes are a part it will be necessary to climb *down* the regulatory hierarchy to the structural genes operating specifically in the cells whose developmental destiny they affect. Sea urchin developmental biologists face just the converse problem: by taking advantage of the experimental accessibility of early differentiated cell types in this embryo, many specific structural genes that are activated differentially early in development have been discovered, and in some cases even sets or batteries of such structural genes. However, in order to understand the mechanism of their activation, it will be necessary to climb *up* the regulatory hierarchy, so as to identify the higher level genes that control the differential activation of these structural genes in embryonic time and space.

A GLIMPSE OF THE REGULATORY ARCHITECTURE OF THE *DROSOPHILA* EMBRYO

Drosophila embryos develop in a manner that is as different from vertebrate embryos as from sea urchin or nematode embryos. There is no fixed cell lineage that relates specific cell types with specific early blastomeres, and there is also no cell migration prior to the time at which the overall pattern of cell fates becomes fixed. For the first twelve cleavages the embryo develops as a syncytium. At the 8th and 9th cleavages most of the nuclei migrate to the periphery, though a small fraction remain inside the egg, within the yolk. After 9th cleavage about 18 nuclei move into the posterior end of the egg and form pole cells, the progenitors of the germ line. The vast majority of the nuclei which are located at the egg cortex are not enclosed by membranes until after 13th cleavage. Excluding the pole cells and the yolk nuclei the embryo then consists essentially of the two-dimensional blastoderm surface, containing about 5,000 cells. The fate map of the cellular blastoderm stage embryo indicates by position exactly which *region* of the blastoderm invariantly gives rise to each of the various tissues and structures of the gastrula (Hartenstein *et al.*, 1985; for reference and details relevant to the following brief summary see review in Davidson, 1986*b*).

Detailed ablation experiments have shown that by the cellular blastoderm stage the *regional fates* revealed by mapping procedures are in fact fixed. However, at least in some regions the individual cell fates are not. Thus, *e.g.*, large bilateral areas on the flanks of the embryo, the ventral neurogenic ectoderm regions, are destined to produce *both* ectodermal cells and neuroblasts, in a ratio of about 3:1. Neuroblast specification appears to occur probabilistically, and judging from experiments on other insect embryos (Doe and Goodman, 1985*a*, *b*) once a neuroblast has been formed it represses surrounding cells from embarking on the neuroblast pathway of differentiation. If an initial neuroblast is ablated, a cell that in the undisturbed neurogenic ectoderm would have expressed a different fate will now become a neuroblast. This form of cell specification mechanism of course requires a particular genetic regulatory structure. Genes are known in *Drosophila* that are required to maintain the proper balance between ectodermal and neuroblast specification (*e.g.*, Artavanis-Tsakonas *et al.*, 1984). Mutations in these genes result in overproduction of neuroblasts at the expense of ectodermal cells. Thus, the function of this developmental genetic system is to maintain the intercellular interaction that results in suppression of neuroblast differentiation by the initial neuroblasts (and hence choice of the ectoderm pathway). Other genes, that function maternally, determine the extent of the ventral neurogenic ectodermal regions. Mutations in certain of these genes cause extension of the neurogenic ectoderm around the whole of the embryo, and mutations in others have the converse effect, *i.e.*, its replacement by dorsal ectoderm, which lacks the capacity to produce ventral ganglionic neuroblasts (Campos-Ortega and Hartenstein, 1985).

A prominent aspect of insect anatomy is metameric structure. At the cellular blastoderm stage the anlage for each of the future metameric units is 3–5 cells wide. Recent experiments have shown that the positions and sizes of the metameric units, and their identity, are both established by processes requiring zygotic gene function, even before cellularization is complete. Mutations in some genes involved in the initial establishment of metameric organization result in gross deletion of contiguous segments, and in others, in deletions of segmental structures that are repeated throughout the larva at intervals of every other segment (Gergen *et al.*, 1986). A particulary interesting and well studied example of the latter is a gene called *ftz*. Molecular and genetic analyses show that this gene codes for a nuclear protein that appears in the cells of the segmental anlagen of those seven alternate metameric units that are missing when the gene is mutated (Carroll and Scott, 1985); that the *ftz* gene interacts with some other genes required for segmental determination, so that the normal patterns of expression of these other segmentation genes are required for the normal pattern of expression of the *ftz* gene (Carroll and Scott, 1986; Harding *et al.*, 1986); and that forced ectopic expression of the *ftz* gene in metameric units where it is not normally expressed disturbs development in those units, just as failure of expression in the alternate units prevents their normal development (Struhl, 1985). That is, both the expression of the *ftz* gene, and the alternating absence of expression, in other words the *overall spatial pattern of expression*, are necessary for normal metamerization. All of these findings indicate *ftz* to represent a class of putative regulatory genes required for cells of the blastoderm to recognize their situation in metameric terms. This process of *territorial designation* is to be distinguished from the institution of cell differentiation, by which subsequently arise the specific cellular structures that in the postgastrular embryo and larva, produce the particular characteristics of each metameric unit. There are so far about 20 genes known to be required for the establishment of metamerization. It is significant that in the regional specification process utilized in the *Drosophila* embryo some of the earliest differential gene activations are evidently required for the delimitation of spatial domains, rather than for the generation of particular differentiated cell types.

The establishment of *segmental identity* is controlled by a different set of genes, mutations in which may result in replacement of morphological patterns characteristic of given segments or parts of segments with those of other segments. The best known are the genes of the *bithorax* (Lewis, 1978, 1985; Hogness *et al.*, 1985) and the *Antennapedia* (Wakimoto and Kaufman, 1981) complexes, but others have been identified as well. Genes of this nature have properties expected of upper level regulatory genes. All such loci so far examined also produce nuclear proteins that are synthesized in certain regions of the embryo (*e.g.*, Beachy *et al.*, 1985), generally those affected by the respective null mutations. These genes also interact with one another, in that the spatial distribution of their products is mutally dependent on normal function (Hafen *et al.*, 1984; Beachy *et al.*, 1985; Levine *et al.*, 1985; Capdevila *et al.*, 1986). The segmental identity genes ultimately determine the disposition of many different cell types, and thus affect the localized expression of many individual structural genes. However, since many of the same cell types occur in different segments, it seems most likely that regulation of these structural genes is controlled at an intermediate hierarchical level.

Both antero-posterior and dorso-ventral axes are predetermined in the *Drosophila* egg, and in fact are structurally evident far back in oogenesis. The initial patterns of function of the zygotic regulatory genes responsible for the spatial organization of the *Drosophila* embryo depend on global positional information resident in the egg. Thus, maternally acting genes are known that derange both axes of this egg, resulting in faulty activation of zygotic genes responsible for morphological pattern formation (Nüsslein-Volhard, 1979; Anderson and Nüsslein-Volhard, 1984). For instance, in eggs derived from females mutant for a gene called *bicaudal*, which as the name suggests give rise to embryos with two posterior ends, the normal pattern of *ftz* gene expression is deranged as well (Gergen *et al.*, 1986; see also, *e.g.*, Carroll *et al.*, 1986). In sum, genetic and molecular analyses have provided examples of genes that mediate and some that probably regulate regional specification at the blastoderm stage, occurring with reference to a preformed, spatial matrix that is initially set up during oogenesis. In the embryo the morphological pattern is materialized, and then refined, into specified metameric units through zygotic gene activity during blastoderm formation. Many steps later, *i.e.*, long after gastrulation, this pattern is translated into a specific and detailed segmental organization of differentiated cell types. The downstream mechanisms by which this is accomplished so far remain almost completely obscure.

CLIMBING UP THE REGULATORY HIERARCHY: AN EXAMPLE FROM RECENT RESEARCH ON SEA URCHIN EMBRYOS

Cytodifferentiation is evident in several cell types of the sea urchin embryo as early as the blastula stage when the embryo contains only a few hundred cells. In the undisturbed embryo these cells are related to the early cleavage founder cells by a fixed lineage (Davidson, 1986*b*; Cameron *et al.*, 1987), but unlike the case of the *C. elegans* embryo, for the majority of the sea urchin embryo blastomeres cell fate is not an autonomous property of the cell lineage. Thus most of the cells remain plastic, their fates apparently dependent on both intercellular interaction and the region of the egg from which they derive, until late in cleavage (Hörstadius, 1939). The egg is endowed at fertilization only with its animal-vegetal axis. The second axis, *i.e.*, the oral-aboral axis, may not be specified until the 8-cell stage (Czihak, 1963; Cameron *et al.*, 1987). At the vegetal pole of the egg there arises the only lineage that is clearly fixed and autonomous from the beginning, *viz.* that giving rise to the primary mesenchyme cells which later secrete the larval skeleton. The sea urchin embryo is a mixed system, in that both interblastomere induction and cytoplasmic localization of maternal factors are evidently involved in blastomere specification. Furthermore, different regions of this embryo develop in different ways. The ectoderm, in which there is no cell migration whatsoever, develops as a series of contiguous clonal patches (Cameron *et al.*, 1987). As do the skeletogenic primary mesenchyme cells, ectoderm cells on the future aboral side display differentiated molecular functions relatively early in development. The internal organs of the larva, on the other hand, develop from a pluripotential set of blast cells held throughout the blastula stage in a disc around the vegetal pole of the egg. At gastrulation these cells invaginate to form the gut, and they then give rise to highly migratory secondary mesenchyme cells, from which derive muscle, pigment cells, coelomocytes, much of the imaginal coelomic rudiment from which the adult sea urchin derives, etc. Differentiated functions in gut and secondary mesenchyme appear only at and after gastrulation. There is no metamerism, and no evident requirement for regional specification of pattern imposed on a previously indifferent set of cells of indeterminate lineage, as in *Drosophila*.

Many sea urchin structural genes are now known that function exclusively in cells of given embryonic lineage and type (see reviews in Angerer and Davidson, 1984; Davidson, 1986*b*). These include genes that are expressed only in muscle cells, pigment cells and gut cells, amongst the vegetal plate derivatives; in the skeletogenic mesenchyme cells; and in the aboral ectoderm. In several cases these genes code for proteins that contribute in an understandable way to the functional properties of the cells in which they function, providing a conceptual bridge amongst gene action, embryonic cell function, and morphology. Thus for example, a gene coding for a structural matrix protein of the embryonic calcite skeletal elements, or spicules, has been cloned and characterized (Benson *et al.*, 1987; Sucov *et al.*, 1987). This gene is expressed only in the skeletogenic mesenchyme cells. In recent studies we have focused on a cytoskeletal actin gene called CyIIIa, which is expressed only in the aboral ectoderm. The actin protein encoded by this gene differs very little from other cytoskeletal actins in the embryo, though the gene is regulated in a distinct manner, except for the closely linked CyIIIb gene, which is also expressed only in aboral ectoderm (Shott *et al.*, 1984; Lee *et al.*, 1986; Akhurst *et al.*, 1987). The ~200 aboral ectoderm cells of the blastula stage embryo descend clonally from six early cleavage progenitors, and except for a very small amount of maternal expression during oogenesis, CyIIIa gene transcription is confined to the descendants of these cells, which are lost at metamorphosis. The gene never functions in postmetamorphosis juveniles. The function of the CyIIIa actin protein is probably to provide the single cell-thick aboral ectodermal wall of the larva with its rigid structure. In addition to the CyIIIb gene, all the members of the Spec gene family, which codes for Ca^{2+} binding proteins, are also activated exclusively in the aboral ectoderm (Lynn *et al.*, 1983). Thus we know of a battery of genes that are differentially expressed in this cell type.

The gateway to causal analysis of differential CyIIIa expression in the early embryo is identification of the regulatory regions of the gene. This is being accomplished by means of gene transfer. We removed the protein coding regions of the CyIIIa actin gene, and replaced them with a commonly used "reporter" sequence, the bacterial gene coding for chloramphenicol acetyltransferase (CAT), which is easily assayed. We found that when this fusion construct is injected into unfertilized sea urchin eggs, and these are then allowed to develop, CyIIIa regulatory sequences direct synthesis of CAT enzyme at exactly the stage of development when the endogenous CyIIIa gene is expressed (Flytzanis *et al.*, 1987). Furthermore, CAT mRNA appears only in aboral ectoderm cells (Hough-Evans and Davidson, 1987). By deleting regions of the upstream sequence, and other more refined experiments, we located the DNA sequences that include the necessary regulatory regions. The facts that the fusion construct works properly in space and time, and that specific contiguous sequences are required, imply their interaction with diffusible activators, *i.e.*, the products of regulatory genes responsible for the differential CyIIIa gene expression. At least some of these have now been identified as well (Calzone *et al.*, 1987), as embryo nuclear proteins that bind with enormous specificity to those upstream regions of the CyIIIa gene that are required for expression.

To solve the specific problem of understanding CyIIIa gene expression in the embryo, and to ascend into the regulatory hierarchy, it will be necessary to obtain molecular probes for these DNA-binding factors, and thereby to follow their distribution or activation in the founder cells of the aboral ectoderm. Presumably the intercellular polarization probably required for the specification of the oral-aboral axis will play a role in this process. An interesting fact emerging from current observations is that most of the sequence-specific proteins that react with regulatory regions of CyIIIa DNA are maternal; that is, they can be found in unfertilized egg cytoplasm (F. Calzone, P. Thiebaud, N. Theze, and E. Davidson, unpublished data). A crucial part of the explanation we seek might thus lie in the mechanism of their axial distribution in the early cleavage embryo.

CONCLUDING REMARKS

I have sought in this essay to indicate briefly the conceptual thrust of the powerful paradigms now being applied to the basic phenomenon of early development. The main features are the idea that spatial regulation of differential gene activity underlies embryogenesis, and the conviction, which is hard to gainsay, that with extant tools the molecular mechanisms by which this occurs can be discovered and defined. The outlines are already evident. The genes that ultimately endow the embryonic cells with their different characteristics and fates are activated by highly specific interactions with diffusible regulatory factors. I believe that the means by which the correct spatial presentation of the necessary regulatory factors is arranged, the levels of hierarchy required, and the functions of the genes that are being regulated, will all turn out to be characteristic and distinct for each system of embryogenesis. It is in unravelling these very distinctions that we will meet, and solve, the real biological problem of explaining embryonic development, in its many forms.

A criticism that some thoughtful (and some not so thoughtful) people have made is that the approach fails because it is too "reductionist." This argument has two main parts: that by the time one knows the molecular mechanisms one must have lost sight of the biologically interesting processes; and that exclusionary focus on one or a few biological models will produce a fallacious description of development, in which biological diversity is ignored, swept under the rug, so to speak, or consigned (*a posteriori*) to the category of trivial phenomenological detail. In my view the first of these arguments is simply without substance, and in fact implies that the field is moving in a direction the opposite of its current real progress. The present focus on the flow of developmental genetic information, and on the spatial and temporal control of embryonic structural gene expression, will lead to mechanistic explanations of just those "biologically interesting" problems that our predecessors described. Among these are localization and induction, which are both at last beginning to be approached in a fresh manner. The second criticism I sympathize with. However, this is not intrinsically a criticism of a "reductionist," molecular level approach, but rather of biological arrogance and ignorance. I have tried to stress the extent to which organisms indeed differ fundamentally in developmental strategy, and the likely ramifications even at the basic level of what I term *regulatory architecture.* To abandon finally the argument between "reductionist" and "holistic" approaches to developmental biology, as an earlier generation finally buried the strenuous discussion between preformationism and epigenesis, is indeed a difficult challenge. It requires that we remain biologically intelligent molecular biologists, who keep our minds on all levels of process, and on the variety of forms of development. This may entail intellectual difficulty on a grand scale, but such perhaps is a requirement for significant advance in scientific understanding.

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Determinants of Early Amphibian Development[1]

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SYNOPSIS. The animal-vegetal organization of the amphibian egg may originate from the axis of organelles and cytoskeletal elements established in the oocyte as it divides from the oogonium. Along this axis, cytoplasmic materials are localized during oogenesis: yolk platelets, for example, are translocated toward the vegetal pole, increasing their amount and size in that region. In the first cell cycle after fertilization, the egg cortex rotates 30° relative to the cytoplasmic core, modifying animal-vegetal organization. The direction of this rotation, biased by the point of sperm entry, defines the site of development of anatomical structures of the dorsal midline of the embryo. As its immediate effect, rotation activates the cytoplasm of a subregion of the vegetal hemisphere, causing cells cleaved from this subregion to be more effective than other vegetal parts in inducing marginal zone cells to initiate gastrulation movements. The most strongly induced part of the marginal zone begins gastrulation first (the dorsal lip of the blastopore) and proceeds through a series of cell interactions leading to its determination as the anterior dorsal mesoderm of the embryo. If these cell movements are inhibited in the gastrula stage, or if vegetal induction is inhibited in the blastula stage, or if cortical rotation is inhibited in the first cell cycle after fertilization, the embryo always fails to develop dorsal structures of the anterior end of its body axis; the more inhibition, the more posterior is the level of truncation, until a radial ventralized embryo develops, derived from the animal-vegetal organization of the oocyte.

INTRODUCTION: REGION-SPECIFIC MODULATION OF STAGE-SPECIFIC MORPHOGENETIC PROCESSES

From the fact that each part of the amphibian embryo arises reliably from an identifiable part of the egg, embryologists for almost a century have considered that the egg cytoplasm must contain spatially-organized instructive materials ("determinants") responsible for embryonic development. To this day, it remains uncertain what sort of determinative effect we must ascribe to these agents. One extreme possibility, mentioned in this volume's introductory chapter (Moore, 1987), is that the variety and organization of these egg materials are very extensive, perhaps equalling the anatomical complexity of the larva itself. By this entirely mosaic viewpoint, organismal development is the aggregate of locally autonomous conversions of egg materials into embryonic materials, without an increase in the complexity of organization. Since it left unsaid what organizes these region-specific determinants so precisely in the first place, the ultimate question of the origin of pattern, and of development itself, is avoided. A modern addition to this mosaic idea holds that all determinants affect gene expression, thereby controlling the local presence of gene products (an idea often traced to Morgan, 1934) which drive each lineage of cells through a sequence of changes leading to a definitive cell type. Stage-specific developmental changes are thus an effect of region-specific determinants.

We will outline a different viewpoint, also quite hypothetical, which seems more consistent with the results of experimental studies of amphibian development. In this view, we assume that determinants act on many cellular functions in addition to gene expression and fall into two categories of determinative effect: One category we will refer to as "determinants of stage-specific morphogenetic processes" and the other as "region-specific determinants." The former would be distributed uniformly in the egg and embryo and would be responsible for the stepwise changes occurring in development. The latter would be non-uniformly distributed and would regionally modulate the former so that developmental change does not occur isotropically in the embryo. The latter determinants would not cause changes to occur but would just modulate spatially the changes that would occur anyway. The morphogenetic process of one stage, when it acts isotropically, would produce region-specific determinants for the next stage, and these in turn would make the next morphogenetic process non-uniform. Thus a non-uniform change at one stage insures that the next change will be non-uniform in a topographically related way. This is an epigenetic, not mosaic, viewpoint since morphogenetic processes, acting anisotropically, increase the complexity of the developing organism in a stepwise manner. Thus the egg would not have to equal the embryo in its organizational detail. This view also differs from the mosaic view in that most parts of the embryo could not develp autonomously from small fragments of the egg: region-specific modulation of development would require the interaction of parts of the egg and embryo.

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In this article, we examine four stage-specific processes of vertebrate development, their region-specific modifiers, and their effect in increasing the embryo's organizational complexity. Two of these processes are intracellular and occur at single-celled stages of development, namely, in oogenesis and in the newly fertilized egg. The other two occur later between cells formed by the mitotic divisions of the egg. Although we do not know the molecular identity of the determinants, we know how they are generated, the time and place of their action, and the consequences of their failure. The example of *Xenopus laevis*, the South African "three-clawed toad," is favorable for use because the egg and embryo develop externally and are large, hardy, and readily available in the laboratory. Background on the development of this amphibian can be found in recent reviews (Kirschner and Gerhart, 1981; Gerhart *et al.*, 1986; Gerhart and Keller, 1986).

PRIMARY POLARIZATION OF THE OOCYTE

The *Xenopus* egg (diameter 1.2 mm) is a highly polarized cell with a brown animal hemisphere and a yellow vegetal hemisphere. The animal half develops into the nervous system and skin of the tadpole, whereas the vegetal hemisphere gives rise to the digestive and respiratory systems. The skeletal, muscular, circulatory, and excretory systems develop from a broad band of cytoplasm at the equatorial boundary of the two hemispheres, a region known as the marginal zone. The blastopore arises in embryogenesis on a latitude line 50° from the vegetal pole. Thus, organ systems of the embryo derive reliably from differentiated cytoplasmic regions of the unfertilized egg. The two hemispheres represent major cytoplasmic localizations established during oogenesis. Though none has been identified, it seems likely that the hemispheres contain different region-specific determinants which modify processes common to both hemispheres.

What process generates the egg's hemispheric organization, that is, its primary polarity? The egg's precursor, the oocyte, grows and differentiates in the ovary of the frog, after arising as a small cell (20 μm diameter) by mitosis from a self-perpetuating stem-cell precursor, the oogonium. Even at this earliest stage, the oocyte is a polarized cell. At one end, a cytoplasmic bridge connects it to sister oocytes. Near the bridge is a centrosome surrounded by numerous mitochondria and Golgi vesicles, and farthest from the bridge is a large nucleus whose chromosomes orient their tips toward the centrosome at an early meiotic stage. Thus, the oocyte may never be without organization; it may become polarized by endogenous means at the time of its division from the oogonium.

The axis of organelles in the young oocyte typifies many eukaryotic animal cells for at least some period in their cell cycle. The oocyte may just preserve and build upon this common organization to differentiate two distinctive hemispheres. In the full grown oocyte, the nucleus does in fact occupy the animal half, while the cluster of mitochondria and of associated germ plasm granules occupies the vegetal pole (Tourte *et al.*, 1981; Heasman *et al.*, 1984). (The cytoplasmic bridge disappears early in oogenesis.) Many materials add to the cytoplasm of one hemisphere or the other during the oocyte's 10,000-fold growth in the ovary—the mass of close-packed, large, membrane-bounded yolk platelets in the vegetal hemisphere, for example, or certain types of mRNAs (Rebagliati and Melton, 1985). We do not know the oocyte's processes for distributing materials to the two ends of its organellar axis, but presumably these are the same cytoskeletal and membrane-mediated processes used by other cell types to localize intracellular materials. For example, the nutritive protein, vitellogenin, is synthesized in the liver of the female frog, circulates in the blood stream, and is taken up equally over the entire oocyte surface by receptor-mediated endocytosis, a process common to most cell types. Vitellogenin collects in membrane-bounded endocytotic vesicles which fuse to form yolk platelets (Wallace *et al.*, 1983). These are translocated toward the vegetal pole where they accumulate, giving that hemisphere its abundance of nutritive material. When new vitellogenin-filled endocytotic vesicles arise at the vegetal surface, they are likely to fuse with the many close-packed pre-existing platelets, increasing the size of these platelets still further. In the animal hemisphere, endocytotic vesicles meet pre-existing platelets less frequently and more often initiate new small platelets. Thus, the animal-vegetal difference of platelet size is a secondary consequence of the difference in platelet abundance caused by directional transport (Danilchik and Gerhart, 1987). The directional transport of platelets into the vegetal hemisphere seems a consequence of the polarized cytoskeletal organization of the growing oocyte. Perhaps this same mechanism is used for the localization of other cytoplasmic materials in the oocyte as well.

Although we will not discuss this point in detail, it should be recalled that the amphibian oocyte stockpiles many biochemical materials for later development and keeps them in an inactive state. For example, there are sufficient nuclear proteins for several thousand blastula nuclei, sufficient mRNA for the late blastula stage, sufficient ribosomes for the tailbud stage of 50,000 cells, and sufficient mitochondria for even later. And yet the functioning of these materials is greatly suppressed in early development. Some of the stage-specific morphogenetic processes of early development probably involve the stepwise activation or disinhibition of the normal function of these various stored materials, until normal somatic cell behavior is achieved. The inhibition of these functions in the oocyte, and their disinhibition in embryogenesis, are little understood but, as a subject of study, these phenomena might plausibly provide information about stage-specific determinants of morphogenetic processes in early development.

SECONDARY POLARIZATION OF THE EGG

Although the unfertilized egg's hemispheres give rise to different organ systems of the embryo, we cannot predict the exact developmental fate of any egg region until after fertilization. A single sperm enters anywhere in the animal hemisphere, where it leaves a dark spot. If we define a meridian connecting the hemispheric poles and running through this spot, this meridian coincides reliably with the eventual ventral midline of the embryo and tadpole, and can therefore be referred to as the prospective ventral midline. The egg's opposite meridian comes to lie on the embryo's dorsal midline, in the region that will give rise to the notochord, nerve cord, and other dorsal structures which characterize vertebrates; this meridian is the prospective dorsal midline of the embryo. These topographic relationsips were appreciated over a century ago by G. Newport and W. Roux, as is described in this volume's introductory chapter (Moore, 1987). Since the sperm enters at random and since these topographic relationships are predictable from its entry point, we can conclude that the sperm in some manner determines the embyro's dorso-ventral organization.

What developmental process does the sperm affect? It is well known that the egg reorganizes its cytoplasm in the first cell cycle after fertilization, and that this reorganization is essential to the development of dorsal but not ventral embryonic structures (Ancel and Vintemberger, 1948; Elinson, 1980). The grey crescent is an external manifestation of this reorganization; it is a region of lessened pigmentation formed at the equatorial site most distant from the sperm entry point, midway in the first cell cycle after fertilization. Various authors have thought that the grey crescent is itself a cytoplasmic localization of determinants of dorsal development since it coincides with the site of the future dorsal blastopore lip of the embryo, that is, the site of the Spemann organizer region.

In the following paragraphs we describe recent experimental findings about this reorganization and the sperm's role as a spatial cue or region-specific determinant of the direction of this morphogenetic process. These experiments extend the classic observations of Ancel and Vintemberger (1948) that the egg cortex and underlying cytoplasm can move relative to one another. In order for us to follow the movements of egg materials, we mark the newly fertilized *Xenopus* egg with an array of fluorescent spots (Vincent *et al.*, 1986). In many cases we selectively stain the subcortical cytoplasm which reaches to within 5 μm of the surface. To facilitate our observations, we then embed the eggs in a gelatin matrix which immobilizes the egg surface. The subcortical cytoplasm begins to move 45 min after fertilization and stops at 90 min (the first cell cycle lasts 100 min) after a displacement by 30° of arc. This is a linear distance of 350 μm in this large cell. The spot array retains its spacing and sharpness as if the cytoplasm is quite rigid. Spot patterns applied anywhere on the subcortical cytoplasm of the egg give similar results. Thus, the entire cytoplasmic core of the egg rotates 30° as a rigid ball relative to the immobilized cortex. The grey crescent is merely a locality where the egg's pigmentation is favorable for seeing this global rotation, which involves the entire egg.

Viewed from the vegetal pole, fluorescent spots on the core are seen to move in a straight line across the pole, roughly away from the prospective dorsal midline defined by the sperm entry point. When eggs are left for a day to develop in the gelatin matrix, we find that the embryonic dorsal midline reliably arises on the meridian away from which the spots had moved a day earlier in the egg. In fact, the direction of spot movement proves to be a much more accurate indicator of the future position of the dorsal midline than is the sperm-defined meridian. We consider it likely that the sperm entry point, or actually the sperm aster originating near the entry point, specifies only vaguely the direction of rotation of the cytoplasmic core, and that this rotation, not the sperm entry point, defines accurately the position of the dorsal midline of the embryo.

Related patterns of movement are obtained for eggs marked with fluorescent spots on the external surface, provided the egg is not embedded in gelatin but allowed to orient itself freely in water, as would be the case for a frog egg developing naturally. The inner cytoplasm of the egg is weighted heavily by the mass of dense yolk in the vegetal hemisphere. When the egg is free to orient itself, the vegetal yolk mass remains in gravitational equilibrium at the lowest position, while the egg surface rotates 30°. The surface movement is equal in magnitude but opposite in direction to that observed for the cytoplasmic core of embedded eggs. Thus, the surface and core move as rigid units relative to one another, and a net displacement is observed for whichever unit is not restrained. Small fragments of the vegetal hemisphere are able to engage in rotation, indicating that the mechano-chemical machinery is available locally in this hemisphere (Vincent and Gerhart, 1987*b*).

Rotation is an interesting geometric operation by which the egg replaces cylindrical symmetry with bilateral symmetry. Since the egg possesses only two moving units, there can be only one direction of rotation. The animal-vegetal polarity of the egg's cortical layer is offset 30° relative to the animal-vegetal polarity of the inner cytoplasm. Whatever materials might be graded or localized in the animal or vegetal portions of the cortex and core, these are brought into new appositions by the rotation to produce a unique bilateral pattern. There seems to be little if any mixing or localizing of materials. Rotation causes a secondary polarization of the egg's structure by systematically displacing layers of the primary polarity. This new pattern of apposed materials is thought to constitute the first dorsal-ventral differentiation of the egg. As discussed later, rotation seems to activate one subregion of the vegetal hemisphere—that region in which subcortical cytoplasm moves farthest in a vegetal direction. Rotation is certainly a morphogenetic process. It is epigenetic in character, for the egg separates its contents into two units which can interact, once moved, to give spatial organization more complex than could have been formed without rotation. Finally, it is entirely a maternal process, with no need for new gene expression. In fact, it is a post-translational process, as shown by the fact that eggs treated with cycloheximide before fertilization (to suppress protein synthesis by 95%) will nonetheless, once fertilized, engage in a normal amount of rotation at the correct time (Vincent and Gerhart, 1987*b*).

What is the role of the sperm as a determinant in secondary polarization? To approach this question, we have followed Ancel and Vintemberger (1948) in studying artificially activated eggs. Such eggs, when activated by electric shock or needle puncture instead of fertilization, begin the early stages of development. To our surprise, these eggs rotate the cortex and cytoplasm relative to one another, approximately on time and to the normal extent. However, rotation occurs in an unpredictable direction—with no relation to the point of needle puncture—whereas it would normally align itself with the sperm entry point of a fertilized egg. Thus, the sperm seems to provide a region-specific determinant used by the egg to choose a particular rotation direction, but the sperm does not provide essential components for the rotation, which is driven entirely by maternal stage-specific determinants. The sperm's region-specific determinant may be produced by the sperm aster arising near the entry point and not by the entry point itself (Manes and Barbieri, 1977).

Is rotation in the absence of the sperm really effective in specifying the dorso-ventral develpment of egg regions? This is not easy to answer since artificially activated eggs fail to cleave, lacking as they do the sperm's microtubule organizing center (*i.e.*, its centrosome) required for spindle formation and successful mitosis. To meet this requirement, we wait until an artificially activated egg has finished its rotation and then we transplant into it a nucleus and centrosome from a single blastula cell.

From our record of the direction of rotation, we can predict where the embryonic dorsal midline should arise, and indeed the prediction has been borne out, irrespective of the site of injection of the centrosome and nucleus. Thus, we conclude that the egg's rotation without the sperm can in fact specify dorso-ventral development. Clearly, the sperm is but a region-specific modifier of the direction of rotation, whereas rotation is a maternal stage-specific morphogenetic process. This is a remarkable example of the independence of a morphogenetic process from the region-specific determinants that can modulate it.

THE ROLE OF ROTATION

Our experimental test of the importance of rotation has been to prevent it, and then to observe the consequences for later development. This simple approach has provided clear answers. There are diverse, easily reversible treatments which inhibit rotation. These include brief cold shock (2°C, 4 min), high hydrostatic pressure (6,000 psi, 6 min), or a low concentration of nocodazole (5 min). The injection of colchicine or vinblastine, though not reversible, stops rotation immediately. All of these reversibly depolymerize microtubules in living cells. Thus we think rotation in some way requires microtubules (Manes *et al.*, 1978; Vincent and Gerhart, 1987*b*). Another effective treatment, which may not affect microtubules, is UV irradiation applied to the vegetal—but not the animal—surface of the egg (Grant and Wacaster, 1972; Malacinski *et al.*, 1977; Manes and Elinson, 1980).

These treatments, when applied to the egg before or during rotation, produce a specific syndrome of develomental defects. The egg cleaves normally and then gastrulates. However, gastrulation is abnormal. It is delayed and cylindrically symmetric in its movements—an important point returned to later. Neurulation never occurs. The embryo develops into an "invertebrate"organism with a simple three-layered anatomy of ciliated epidermis, red blood cells, coelomic chambers, and a short gut. It has no head, trunk, or tail, and no dorsal structures. After a few weeks it dies when its yolky food reserves are exhausted. Thus, the egg's failure to rotate during the first cell cycle means that the cylindrically-symmetric primary polarity of the oocyte persists throughout embryonic development. We have thus succeeded in eliminating a whole sequence of region-specific modifiers, all dependent on rotation for their formation, while leaving many stage-specific morphogenetic processes intact. This is of course very valuable for the experimental identification of the two types of determinative effects.

From the type of gastrulation behavior and the types of final embryonic tissues formed by non-rotating eggs, we conclude that the "limit form" is in many respects a ventralized embryo. Ventral development seems to be a default pathway, or ground state, available to egg regions even in the absence of rotation, that is, without the intervention of region-specific determinants produced by rotation. Development of embryonic dorsal structures depends entirely on the rotation of the egg's internal materials in a vegetal direction. In this sense, dorsal development is a step beyond ventral development. It is interesting in this regard that dorsal structures, such as the notochord and neural tube, are the ones most characteristic of vertebrates.

The intermediate forms of embryos are also interesting. These are obtained when rotation of the egg is incomplete, for example, 5°, 10°, or 15°, instead of 30° of arc. When the cytoplasmic displacement is small, the embryo develops a tail but otherwise lacks vertebrate structures. After intermediate displacements in the egg, the embryo develops a tail and trunk, but no head. And finally at greater displacements, it develops a tail, trunk and head, with hindbrain, midbrain, and forebrain structures added in that order. Thus, we reach the important conclusion that whereas the *direction* of rotation specifies the position of the dorsal midline, the *extent* of rotation specifies the antero-posterior succession of structures on that midline. As described before, the sperm modifies the direction of rotation; however, it seems to have no control over the extent of rotation or over the egg's magnitude of response to the rotation, a point discussed later. In summarizing the importance of rotation, we can say that long before embryonic gene expression begins, the egg engages in a stage-specific process that modifies its cytoplasmic materials in a systematic region-specific way. Rotation, being necessarily unidirectional, inevitably produces regional cytoplasmic differences. Without rotation, the nervous system, skeletal system, and musculature, with all their specific patterns of gene expression, are never initiated.

ARTIFICIAL ROTATION

We can further examine the importance of rotation by causing it to occur artifically in eggs inhibited for natural rotation. Since the egg's inner core of cytoplasm is weighted in the vegetal hemisphere by dense yolk platelets, it will move along the cortex if the egg is kept out of gravitational equilibrium—with the animal-vegetal axis horizontal, for example. Thus, gravity can be used to rotate cytoplasmic materials artificially (Penners and Schleip, 1928). This artificial rotation has many of the effects of the normal one. It can override normal rotation and specify the meridian at which the egg will develop its embryonic dorsal midline (Kirschner and Gerhart, 1981; Gerhart *et al.*, 1981; Black and Gerhart, 1985). More surprisingly though, if eggs are first treated with UV irradiation, cold, pressure, or nocodazole to inactivate the normal cellular mechanism of rotation, and are then kept out of gravitational equilibrium for 40 min, they are completely rescued (Scharf and Gerhart, 1980, 1983). The extent of rescue depends on the length of time they remain out of gravitational equilibrium: If eggs are turned upright after only 10 min, they develop a tail but not a trunk or head. If 20 min are allowed, they develop tail and trunk; and if 30 min are allowed, they develop tail thru midbrain. The body axis forms along the meridian of the egg that was highest in the gravitational field during the period of oblique orientation, that is, on the meridian along which internal cytoplasmic materials slip farthest in a vegetal direction.

Centrifugal force can accomplish an equivalent displacement of egg materials within a few minutes. If we centrifuge the egg twice in opposite directions, we can displace materials twice along opposed meridians of the egg, with the result that many twin conjoined embryos develop (Black and Gerhart, 1986). These have two complete sets of dorsal structures and a single shared set of ventral structures.

It is remarkable that these artificial movements suffice for the development of a normal embryo, one as well patterned as by the egg's own mechano-chemical force-generating mechanism. To us this indicates that the developmental effects of rotation are not really force-specific; cytoplasmic displacements provoked by any means will probably produce the region-specific determinants needed for embryonic axis formation. The egg probably has the potential to form the dorsal midline at any meridian of its circumference, and rotation—which can go in any vertical direction—may simply activate (or disinhibit) the use of this potential on the single meridian along which subcortical cytoplasm has been most displaced in a vegetal direction. According to this model, remaining meridians preserve their potential, and a second cytoplasmic displacement can activate one of these, as in the twice-centrifuged eggs which develop into twins. In distinguishing two types of determinants, we can say that rotation as such is caused by stage specific determinants of the egg, but that the direction of rotation is affected by region-specific determinants introduced by the sperm. Rotation proceeds anisotropically and activates new region-specific determinants in one part of the egg. These in turn affect the spatial character of a later stage-specific process.

Recent experiments indicate to us that the egg can vary the amount of activation that occurs in the vegetal hemisphere cytoplasm as a result of a defined amount of subcortical rotation. Surprisingly, when eggs are treated with 70% D_2O between 25 and 30 min postfertilization, that is, at a time before rotation begins, they can later develop a full body axis with as little as 5 to 10 degrees of rotation. Without D_2O pretreatment of the egg, this small displacement would only suffice for the development of a tail, or a trunk plus tail (Scharf *et al.*, 1984; Vincent and Gerhart, 1987*a*). With larger amounts of rotation, the treated egg actually engages in excessive dorsal and anterior development. The resulting series of embryos is continuous in morphology and dose-dependent on the concentration and duration of D_2O treatment, with extreme forms having large heads and hearts but no trunk or tail, and "limit forms" having radial suckers and eye pigment, and a large central heart. These seem to be the anatomical opposites of the ventralized embryos produced when rotation is inhibited, this new type overrepresenting the dorsal parts of the fate map while diminishing the ventral parts. The existence of these dorsalized forms demonstrates that the egg has the latent capacity to develop dorsally at all positions around its circumference, even though it normally activates only one position. D_2O somehow sensitizes the egg so that rotation activates a much wider arc of positions to make use of this intrinsic capacity. Whatever the exact mechanism, it is striking that the amphibian egg has a morphogenetic process, as well as a response to it, which together allow it to generate such a wide range of anatomies, even though it normally only makes use of a narrow center portion of the range. There may exist a continuous single quantitative variable underlying vertebrate embryonic pattern, rather than a large collection of individual and independent variables for small aspects of pattern.

INDUCTION OF GASTRULATION

The egg's rotation is completed within 90 min after fertilization, shortly before the egg divides to two cells. The egg then divides every 30 min for 12 cycles to produce a spherical liquid-filled blastula of 4,000 cells which initiate gene expression for the first time, 8 hr after the egg's fertilization (Newport and Kirschner, 1982). The time of initiation of gene expression seems the same in both the animal and vegetal hemispheres, and results from the exhaustion of some maternal cytoplasmic material needed for rapid cleavages. Apparently, the mere slowing of the cell cycle, to produce intervals that are neither mitotic (M-phase) nor DNA-synthetic (S-phase) is sufficient to allow the initiation of gene expression (Kimelman *et al.*, 1987). This transition is a clear example of a stage-specific process initiated throughout the egg without respect to region.

Two hours later (at 10 hr postfertilization), gastrulation begins. The cells mainly responsible for gastrulation are located in the marginal zone, a wide band between a latitude line 50° from the vegetal pole, marked by the blastopore, and a second latitude line 50° from the animal pole. Gastrulation is spatially differentiated in its time and intensity, beginning vigorously at the prospective dorsal midline and spreading laterally through the marginal zone with declining intensity, finally reaching the prospective ventral midline two hours later. The early, vigorous region of the marginal zone is the Spemann organizer region which later induces the neural plate and eventually differentiates into embryonic head mesoderm and notochord. The two hour interval before gastrulation is an important period of determination of the time, place, intensity, and type of motility to be exhibited by marginal zone cells.

We will now discuss how the egg's rotation in the first cell cycle determines the temporal and spatial pattern of gastrulation many hours later. As mentioned previously, if the egg's rotation is blocked, the egg cleaves and gastrulates, but gastrulation lacks temporal differentiation; it begins late, weakly, and simultaneously around the entire latitude line. Thus it resembles the normal ventral type of gastrulation. Clearly, the egg's rotation is needed for the embryo to achieve, not gastrulation as such, but an early, vigorous, dorsal type of gastrulation.

We have studied the effect of rotation on gastrulation, by a series of cell transplantation experiments (Gimlich and Gerhart, 1984). These are based on the classic transplantation studies of Nieuwkoop (1973, 1985) and his colleagues by which the stage-specific process of vegetal induction was discovered. They showed that vegetal cells in the two hour period before gastrulation, induce marginal zone cells to acquire their gastrulation activities. In our studies, we blocked rotation in a set of eggs and let them cleave to the 64-cell stage. We then removed two neighboring cells chosen at random from the most vegetal tier of cells of each of these embryos, and replaced them with two cells taken from the prospective dorsal midline of the vegetal tier of a normal embryo of the same age. The operated hosts continued cleaving and in many cases, to our surprise, gastrulated normally and developed a complete vertebrate body axis with head, trunk, and tail, instead of forming a cylindrically symmetric "invertebrate" embryo. In cases of partial rescue, the hosts formed posterior but not anterior elements of the body axis, just as did embryos from eggs with incomplete rotation. The only vegetal cells that promote rescue are those from the prospective dorsal midline, that is, those cleaved from a specific subregion of the vegetal cytoplasm of the egg, the subregion activated by vegetally-directed subcortical rotation in the first cell cycle. Thus we conclude that the dorsal-most subregion of the egg's vegetal cytoplasm is that region activated by rotation.

In order to test whether progeny of the graft cells differentiate into the dorsal structures of the rescued embryos, we repeated the transplantation experiment using graft cells injected with a lineage tracer. This is an inert fluorescent material too large in size to diffuse into neighboring cells by way of gap junctions. In the rescued embryo the direct descendents of the original two graft cells can therefore be easily recognized. We found that the dorsal anatomical structures of the body axis, such as the notochord, muscles, and nerve cord, did not contain any descendents from the graft cells, but only ones from the host. This is remarkable since the host cells would not have formed any of these dorsal structures if the graft cells had not been introduced. Fluorescent descendents of the graft pair were confined to the gut. The graft cells clearly *induced* nearby host cells to develop the entire vertebrate body axis, including the Spemann organizer region and its derivatives, whereas the host cells themselves were not competent to engage in axis formation (Nieuwkoop, 1973, 1985). This special vegetal subregion is sometimes called "the organizer of the organizer." We conclude that subcortical rotation in the egg affects the vegetal cytoplasm of one subregion so that it later, once cellularized, is able to induce the formation of the vertebrate body axis. Although vegetal cells are locally specialized, the animal hemisphere cells are all equally competent to respond and undertake dorsal development: any animal hemisphere cell transplanted into the vicinity of these vegetal cells is activated to express early, vigorous marginal zone activities, to become the Spemann organizer, and to develop dorsal axial structures (Gimlich, 1986).

During the many steps between the grafting operation and the final differentiation of embryonic structures, when does vegetal induction take place and what is its primary effect? Since rescue is obtained even when vegetal cells are grafted into a defective host just 2 hr before gastrulation (Gimlich, 1986), we think the late blastula period is sufficient. Progeny of the special vegetal graft cells remain just below the 50° latitude line of that hemisphere and do not themselves engage in gastrulation movements, whereas host marginal zone cells directly above the graft begin gastrulation early and vigorously—they achieve a dorsal type of gastrulation. We certainly cannot conclude, though, that the special vegetal cells cause gastrulation as such, for as mentioned before, non-rotated eggs are able to undertake a radial ventral-type of gastrulation. Even this type of gastrulation requires a kind of vegetal induction: ventral-most vegetal cells must act on their marginal zone neighbors before these can gastrulate even weakly and late. Induction by ventral-most vegetal cells does not depend on rotation, and it seems less intense or briefer in duration than that imposed by dorsal-most vegetal cells, the ones specialized by rotation. For comparison, animal hemisphere cells which experience no induction at all (*e.g.*, those at the animal pole), only engage in epiboly at gastrulation. Thus, in the late blastula stage, after the midblastula transition, all vegetal cells being to induce marginal zone cells to prepare for gastrulation. However, the dorsal-most vegetal cells are more effective and induce their neighbors to begin gastrulation first and most vigorously.

To summarize the sequence of stage-specific processes and their regional modifiers thus far, we can say the egg is scheduled to rotate its cytoplasmic contents in the first cell cycle and to initiate vegetal induction in the late blastula stage. Vegetal induction will occur even when rotation is prevented. However, the egg's rotation activates one subregion of vegetal cytoplasm which is then incorporated into a subset of vegetal cells during cleavage. These vegetal cells, because of their specialized maternal materials, are more active in induction, and they induce marginal zone cells to start gastrulation vigorously and early, 2 hr ahead of the cells on the prospective ventral midline. When rotation is partially inhibited, induction is diminished, and marginal zone cells begin gastrulation less early and vigorously. When rotation fails altogether, there is still a basal level of vegetal induction, enough for gastrulation to have ventral characteristics. This basal level of induction is intrinsically available to vegetal cells as a stage-specific process, just by virtue of their containing vegetal cytoplasm from oogenesis. Thus, rotation serves to enhance this basal activity locally, making it anisotropic. Rotation allows a region-specific modulation of a later intrinsic stage-specific process. However, rotation does not "cause" induction to happen any more than the sperm entry point "causes" rotation to happen. As stage-specific processes, rotation and vegetal induction must be established by independent determinants in the egg.

We can further ask whether induction actually causes marginal zone cells to undertake gastrulation activities, or only to modify an activity they were scheduled to undertake anyway. Non-induced animal hemisphere cells flatten and spread in "epiboly"; this seems to be a basal level of gastrulation activity intrinsically available to these cells as the consequence of the animal hemisphere cytoplasm they contain, their heritage from oogenesis. Even in small fragments isolated soon after fertilization, animal hemisphere cells can proceed through cell division, blastocoel formation, the midblastula transition, and epibolic expansion, eventually to form a ciliated epidermis rather like normal belly skin of the tadpole. This is a demonstration of the ability of cells to carry out a series of morphogenetic processes on an intrinsic schedule, as the result of the egg's uniformly distributed cytoplasmic determinants of these processes. Thus, while vegetal induction is a very important stage-specific process for determining the types and timings of motile activities of animal hemisphere cells, it may be playing upon (activating or disinhibiting) an intrinsic, pervasive, stage-specific predisposition of animal hemisphere cells to become motile anyway. The region-specific differences in the intensity of induction by the normal vegetal hemisphere, as the result of rotation, insure that the full spectrum of gastrulation motile activities will be present in the animal hemisphere, in a specific region near the vegetal hemisphere, namely, the marignal zone.

Formation of the Vertebrate Body Axis

The fourth and final process to discuss is gastrulation itself, which transforms the organization of the egg into that of the vertebrate embryo. It produces the body axis. In the gastrula, populations of migrating cells of the marginal zone converge toward the prospective dorsal midline, increasing the tissue mass there relative to the ventral midline. Convergence is strongest in the Spemann organizer region. These converging populations pack into extended arrays, lengthening the embryo in the antero-posterior dimension (Keller *et al.*, 1985). As mentioned before, marginal zone cells are directly responsible for forming the body axis in the morphogenetic process of gastrulation.

There is a strong correlation between the time at which cells initiate gastrulation and their final anatomical differentiation. We have alluded to this several times already. The earliest gastrulating cells normally become anterior-dorsal structures, such as head mesodermal and endodermal tissues. The more gastrulation is delayed, the less anterior and dorsal are the final structures, and when it is delayed everywhere to the time normally characteristic of the ventral midline, the embryo develops no body axis at all. This relationship holds for many experimental conditions—for embryos developing from eggs failing rotation to different degrees, or from related eggs that have been rescued to various degrees by gravity or by cell transplantation.

Why does early, vigorous gastrulation lead to dorsal anatomical differention? We have only recently begun to study this question and our results are still tentative. We think that early cells have the longest time to participate in gastrulation, for example to escape from their original associations with neighboring cells, and to complete a series of developmental steps required for subsequent anterior-dorsal differentiation. Cells with less time complete fewer steps and differentiate as more posterior and ventral structures. The number of steps completed by a cell may determine its differentiative fate. This interpretation is consistent with the correlation of the time of gastrulation and the type of differentiation, but more significantly it is consistent with a recent experiment in which we have stopped gastrulation prematurely. We have allowed eggs to begin gastrulation normally, and then have arrested their further progress at various times. For example, the teratogenic dye, trypan blue, very effectively stops the cell movements of gastrulation (Waddington and Perry, 1956). If these movements are stopped rather late, the eventual embryo is headless but the trunk and tail develop normally. The earliest gastrulating cells, which started on time, differentiate as part of the trunk. If gastrulation is stopped somewhat earlier, only a tail is formed and it contains the earliest cells. With still earlier blockage, no body axis develops and the embryo resembles closely the invertebrate forms arising from eggs arrested in their rotation. Thus, preliminary results indicate that the quantity of gastrulation completed by cells somehow determines their antero-posterior and dorso-ventral differentiation (Gerhart *et al.*, 1986; Suzuki *et al.*, 1984). The body axis is progressively truncated from the anterior end as gastrulation stops earlier or starts later. Both departures from normal gastrulation have the same result. For this reason we think cells become ever more anterior and dorsal the longer they engage in gastrulation. As a relevant aside, embryologists have long realized that embryonic cells are not irreversibly determined at the start of gastrulation since there is extensive pattern regulation after dorsal lip grafts into the ventral marginal zone or after ligation of the embryo into lateral halves (Spemann, 1938). Most of the determination of antero-posterior and dorso-ventral fates of cells occurs during gastrulation, not beforehand.

This provisional interpretation allows us to relate the processes of rotation, induction, gastrulation, and axis formation, as follows. Rotation would activate region-specific determinants in a subregion of the vegetal hemisphere. These agents would increase the inductive activity of cells of that subregion, above the intrinsic basal level of other vegetal cells. At the mid and late blastula stages, the vegetal cells induce neighboring animal hemisphere cells to take on motile activities above the basal level of epiboly, and these induced cells form the marginal zone. The most strongly induced marginal zone cells, which of course lie above the most inductive vegetal cells, begin gastrulation first, and because of their earliness they have sufficient time to proceed through the developmental steps needed for anterior-dorsal differentiation, which they subsequently express. The processes of rotation and induction merely establish the spatial and temporal characteristics of gastrulation, but do not determine in any direct way the actual dorso-ventral and antero-posterior differentiation of marginal zone cells. Thus, each developmental stage performs a specific morphogenetic process which augments the anisotropic organization of that stage. At the early stages, a morphogenetic process occurs on schedule even if an earlier process is blocked and even if region-specific determinants are absent, but then the process is isotropic and generates a less rich array of regional differences, *i.e.*, less complexity of organization. However, each process can affect the subsequent process by leaving in its wake region-specific determinants which modulate locally the time, intensity, direction, or position of the subsequent process. The anisotropic operation of these processes increases the embryo's complexity step by step. Since region-specific determinants arise sequentially in regular topographic relationship to one another, the parts of the embryo retain a regular spatial correspondence to the parts of the egg.

Acknowledgments

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Cell Lineages and Determinants of Cell Fate in Development[1]

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SYNOPSIS. The cell lineage phenomenon in ascidian embryos appears to be based in large part on the occurrence of maternally derived egg cytoplasmic determinants. These factors are localized in certain cytoplasmic regions of the zygote and are differentially segregated during early cleavages into specific later tissues and regions of the embryo, where they presumably play some role in establishing the selective developmental fate of larval tissues. Commitment to muscle differentiation is correlated with segregation of a discrete "myoplasmic" region of the zygote: developmental autonomy (self-differentiation) of isolated muscle lineage cells confirms a very early commitment to the restricted fates suggested by the classic cell lineage map. Transfer of myoplasm to other cell lineages by artificial displacement results in some conversion of those cells to muscle expression. Larval muscle acetylcholinesterase, one of the transformation markers used, originates from newly synthesized acetylcholinesterase mRNA at gastrulation; this mRNA first appears only in muscle lineage cells. Indirect evidence suggests that the muscle determinant is a positively acting control factor related to the expression of this and other muscle genes.

INTRODUCTION

In recent decades there has been a remarkable resurgence of research interest in the embryonic cell lineages of animal species, particularly those which have served well as model systems of development for other reasons. This classic problem has been reexamined with up-to-date techniques in the expectation that some new insight might be gained into the mechanisms by which early embryonic cells make restrictive commitments in the fate of their progeny cells. The animal zygote is a pleuripotential single cell which gives rise to a whole organism. Relatively soon in their history the early dividing cells become progressively more restricted in their actual fate, that is, the progeny of particular early cells contribute only to very specific and limited later tissues and organs. This process of early selectivity or commitment has been called "cell determination" in the classic literature; its physiological basis remains one of the great unsolved mysteries of development.

Ascidians (subphylum Urochordata, class Ascidiacea) were among the very first organisms in which cell lineages were studied. As early as 1884, two Belgians, van Beneden and Julin, examined the cleavage patterns in five species of ascidian. They followed the cells as far as the 44-cell stage and established the history of particular cells in relation to their fates as parts of future germ layers of the embryo. In doing so, they observed that cells in the cleavage patterns bore a constant relationship to the original axes of the zygote; these axes in turn had a fixed relationship to the axes of the gastrula and larva.

At a later time Conklin (1905*a*) traced these lineage correlations even further and showed that certain distinct cytoplasmic regions of the zygote were inherited by particular parts and tissues of the developing larva. His work suggested with elegance and clarity that every cell in the division pattern of the early embryo had a specific and possibly invariate fate that was related to its cytoplasmic composition.

Lineage observations in certain species thereby supported a theory of cell determination which asserts that embryonic cells are altered in their commitment towards specific later differentiation by the particular cytoplasmic regions of the zygote which they inherit during cleavage. This is especially true for invertebrate larval development of which ascidian embryogenesis is the classic example. As Stent (1985) pointed out, differential segregation of cytoplasmic agents is only one important meaning of cell lineage. A predictable fate of cells in a lineage also arises from the manner in which division patterns and their consequent mechanics bring about an orderly topographic placement of cells in the embryo relative to other cells and tissues which exert "inductive" interactions. Even in ascidian larval development where the cytoplasmic segregation aspect of cell fate appears to be quite extreme, there is experimentally demonstrable governance of developmental cell fate by inductive cell interactions. The mixture and interplay of these two aspects of development temporally as well as topologically may have many different consequences in regulating cell fate and could explain much of the puzzling diversity observed in cell lineage studies of the various other model species of animal development.

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The present summary uses ascidian embryos to restate and reexamine the evidence, classic and modern, upon which the cytoplasmic determinant theory of cell determination is based. As noted above, this should be considered only one possible aspect of embryonic cell commitment. The theory postulates discrete agents in the zygote cytoplasm arising during oogenesis and which are positioned anisotropically in the egg and zygote. During cleavage, these localized agents which have been variously called morphogens or cytoplasmic determinants, become differentially segregated into certain cells and are thereby passed into specific regions of the embryo. They presumably function at some later time to initiate quite specific differentiation activities in the cells inheriting them.

CELL LINEAGE AND CYTOPLASMIC SEGREGATION

Conklin's (1905*a*) most important contribution to the problem of cell lineage was his finding that certain colored or otherwise identifiable regions of the ascidian zygote cytoplasm were segregated unvaryingly into specific individual cells of the early cleavage pattern. This was particularly obvious in *Styela partita* in which a yellow crescent of subsurface cytoplasm was formed by cytoplasmic rearrangements taking place shortly after fertilization; the crescent material subsequently became segregated during cleavage into the muscle cells of the larva. Conklin and others soon became aware that this "myoplasm" contained a very large number of mitochondria. Contemporary estimates suggest that about two-thirds of the zygote mitochondria are localized in the crescent region of cytoplasm (Berg, 1956; D'Anna, 1966; Whittaker, 1983). Conklin (1931) eventually did centrifugation experiments on the uncleaved zygote in which he displaced the mitochondria from their surrounding myoplasm and found that later cells containing the myoplasm rather than the excess mitochondria differentiated histologically as muscle.

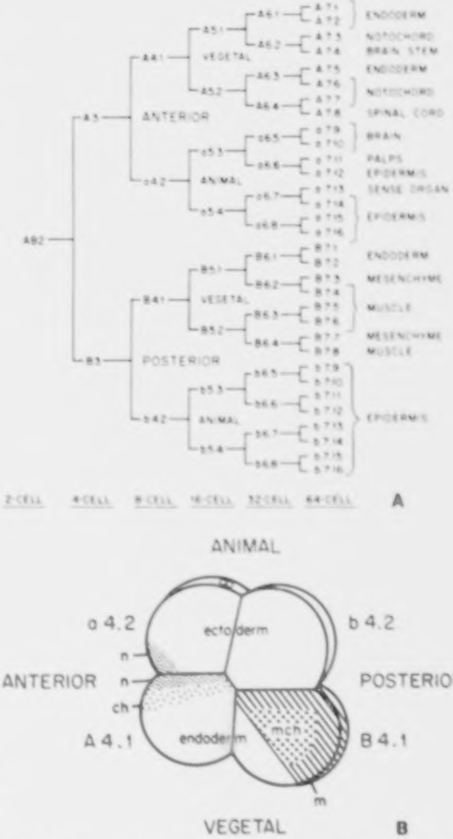
There were also other more subtle regional cytoplasmic differences (shades of color, granularity of the cytoplasm, relative yolk content) in both the *Styela* and *Ciona intestinalis* zygotes. As Conklin (1905*a*) noted, these cytoplasmic regions were themselves apportioned into very specific early cells in a manner similar to the myoplasmic segregation. Different investigators over the years have described additional species with colored plasms (*e.g.*, Berrill, 1948), but it was apparent from histological sectioning and staining of an otherwise colorless and transparent embryo (*Phallusia mamillata*) that there was a mitochondrial-rich myoplasmic crescent, and other distinct cytoplasmic regions that were segregated differentially (Conklin, 1911).

Conklin's (1905*a*) tracing of the cell lineages, by both continuous observation of the living embryos and by sequential fixations of whole embryos, to approximately the 218-cell stage, enabled him to construct a lineage or "fate map" of the bilaterally symmetrical ascidian embryo up to the 64-cell stage with considerable accuracy (Fig. 1A). This map was amended slightly from the results of various cell marking experiments by Ortolani (see Reverberi, 1961) using carbon and chalk particles. The more sensitive and elegant recent investigations of ascidian cell lineages by marking particular blastomeres with microinjections of horseradish peroxidase (Nishida and Satoh, 1983, 1985) have revealed relatively few errors in the Conklin-Ortolani map. These minor differences concern secondary lineages of a few of the distal tail muscle and notochord cells, and will be discussed in a later section of this paper.

An interesting point to emerge from the lineage map, aside from the supposition that lineages may indicate an irreversibly fixed fate of the cells, is that ascidian cells become committed to their ultimate tissue and organ fates quite early in development. Most of them seem to have done so by the 64-cell stage, and only a few cells continue to have mixed fates thereafter, perhaps only to the 128-cell stage, as suggested by Nishida and Satoh (1983, 1985).

From a combination of Conklin's various observations of plasmatic segregation and Ortolani's (1958) marking of various aspects of the egg surface and early cleavage blastomeres with carbon particles, one can reconstruct a map of cytoplasmic territories for the 8-cell stage depicted in Figure 1B. One perceives that segregation of the various "organ-forming substances" in these regions could be responsible for the ultimate fates of the cells indicated by the lineage map (Fig. 1A).

The major point at issue with cell lineage maps is whether they actually do demonstrate the acquisition of irreversible and limited fates by the cells. Are they always, often, or even ever commitment pathways? They certainly indicate what the cells normally become *in situ* during undisturbed circumstances, but whether the cells might actually be able to express other properties remains unresolved by pure lineage tracings.


AB2
A3
ANTERIOR
A4.1
VEGETAL
A5.1
A5.2
A6.1
A6.2
A6.3
A6.4
A7.1
A7.2
A7.3
A7.4
A7.5
A7.6
A7.7
A7.8
ENDODERM
NOTOCHORD
BRAIN STEM
ENDODERM
NOTOCHORD
SPINAL CORD
a4.2
ANIMAL
a5.3
a5.4
a6.5
a6.6
a6.7
a6.8
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a7.10
a7.11
a7.12
a7.13
a7.14
a7.15
a7.16
BRAIN
PALPS
EPIDERMIS
SENSE ORGAN
EPIDERMIS
B3
POSTERIOR
B4.1
VEGETAL
B5.1
B5.2
B6.1
B6.2
B6.3
B6.4
B7.1
B7.2
B7.3
B7.4
B7.5
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B7.7
B7.8
ENDODERM
MESENCHYME
MUSCLE
MESENCHYME
MUSCLE
b4.2
ANIMAL
b5.3
b5.4
b6.5
b6.6
b6.7
b6.8
b7.9
b7.10
b7.11
b7.12
b7.13
b7.14
b7.15
b7.16
EPIDERMIS
2-CELL
4-CELL
8-CELL
16-CELL
32-CELL
64-CELL
A
ANIMAL
a 4.2
b 4.2
ectoderm
n
ANTERIOR
n
POSTERIOR
ch
A 4.1
endoderm
mch
B 4.1
m
VEGETAL
B

FIG. 1. A. The Conklin-Ortolani cell lineages for ascidian embryos to the 64-cell stage; cells from one-half of the bilaterally symmetrical embryo are represented. B. Territories of cytoplasmic fate at the 8-cell stage, mapped according to Ortolani (1954). Abbreviations are muscle (m), mesenchyme (mch), nervous system (n), and notochord (ch).

LINEAGE CELL POTENTIALITY

From the very beginnings of experimental embryology investigators were questioning the actual potentialities of specific early cells in the embryo (Churchill, 1973). There were a number of early attempts to discover the potentiality of ascidian blastomeres, most notably the studies by Chabry (1887) and Conklin (1905*b*, 1906).

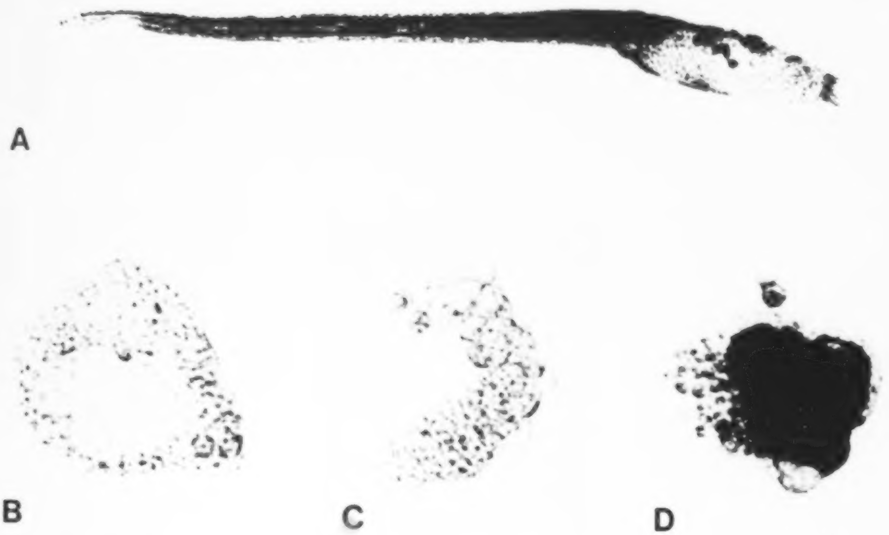

FIG. 2. Histochemical localizations of acetylcholinesterase activity in whole and partial larvae of *Ciona intestinalis*. A. Normal hatched larva. ×110. B. Partial larva from animal half-embryo. C. Quarter larva from A4.1 cell pair. D. Quarter larva from B4.1 cell pair. Magnifications ×420.

These initial studies involved ablation of one blastomere at the 2- and 4-cell stages and did demonstrate an apparently restricted potentiality of the remaining cells. Ablation experiments can be somewhat uninteresting because they lead at best to essentially negative results; in fact, these ascidian experiments did raise many questions about the effect of an injured or dead cell attached to the living ones.

Lillie (1929) made the point quite clearly that we have only one radical criterion of whether the events of early cleavage have actually established the prospective fate or determination of a blastomere. That is, whether those cells or blastomeres, when isolated from the environment and influence of their surrounding cells, express the same fates to which we have assigned them on the basis of other indirect evidence, and whether under such "*in vitro*" circumstances they can express other fates as well. This expectation is reasonable since the embryonic cells of ascidians, and those of many other invertebrates, apparently subsist on their own internal yolk reserves during development. Lillie's rule then is the criterion of autonomous development or "self-differentiation." It is still our only rigorous proof of cell commitment. One also thereby attaches major significance to positive results.

The correspondence between fate maps and the actual potential of ascidian cells became best resolved by the work of Reverberi and Minganti (1946, 1947) although several earlier investigators contributed interesting and important results to the problem. Their studies involved isolations of the bilaterally symmetrical blastomere pairs at the 8- and 16-cell stages, and the finding that such partial embryos readily and only expressed the fates designated by the lineage maps.

Because the Reverberi studies have been based mainly on histological patterns of tissues and organs and histological staining characteristics of certain tissues rather than readily identifiable organelles and cell structures, my co-workers and I have repeated most of the experiments, but using more specific ultrastructural (Crowther and Whittaker, 1983, 1984, 1986*a*, *b*) and histochemical markers of cell and tissue differentiation (Whittaker *et al.*, 1977; Meedel *et al.*, 1987; Whittaker, 1987). Our conclusions differ in no significant way from the original findings of Reverberi and Minganti. Reverberi and Minganti did find that some structures, notably the brain, did not undergo autonomous differentiation but required cell associations with other "inductive" tissues that could be manipulated experimentally. Those experiments have also been reproduced successfully (Whittaker, in preparation).

In extending the earlier analyses of ascidian larval histodifferentiation we have employed quite specific enzymatic and ultrastructural features, mainly because such characteristics of terminal differentiation lend themselves to interpretative associations with specific structural gene activities. Individual proteins and "simpler" macromolecular assemblages are also more easily perceived as an unequivocal end product of cell commitment. For purposes of this brief review, I will concentrate mainly on larval muscle differentiation, about which we now have the most extensive evidence for the occurrence of an egg cytoplasmic determinant. The histodifferentiation markers employed are an acetylcholinesterase enzyme found only in larval tail muscle during embryogenesis (Fig. 2A), as first described by Durante (1956), and the myofilaments and myofibrils of muscle cells (Pucci-Minafra and Ortolani, 1968), illustrated in Figure 3A.

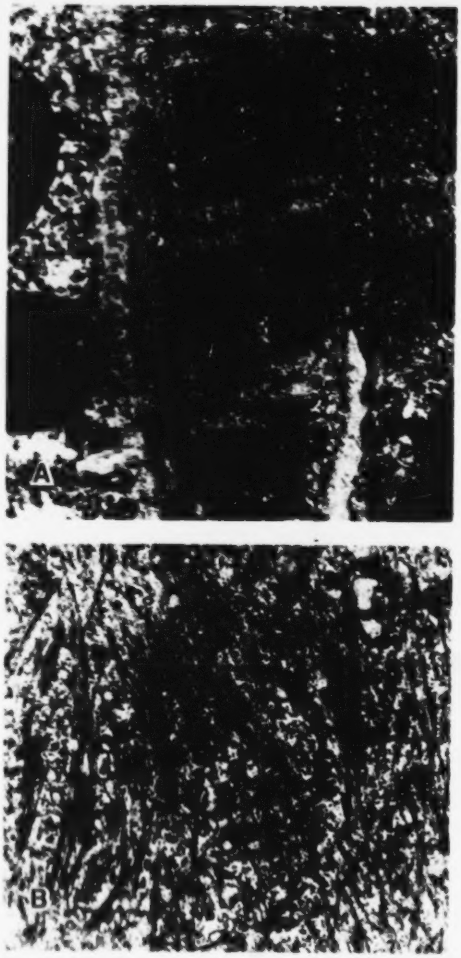

FIG. 3. Transmission electron micrographs of differentiated *Ciona* larval tail muscle. A. 17-hr normal larva. ×38,000. B. Myofilaments and myofibrils in a B4.1-derived quarter-larva. ×16,250.

DEVELOPMENTAL AUTONOMY OF MUSCLE LINEAGE CELLS

The first two cleavages of the ascidian egg occur perpendicular to one another in the plane of the zygote's animal-vegetal axis. These cleavages result in a square of two pairs of contiguous cells arranged on either side of a plane of bilateral symmetry. Third cleavage is then equatorial across the common axis of these four blastomeres to form upper (animal) and lower (vegetal) quartets of cells (Fig. 1B). According to Conklin's (1905*a*) observations on yellow crescent segregation and all later cell lin-

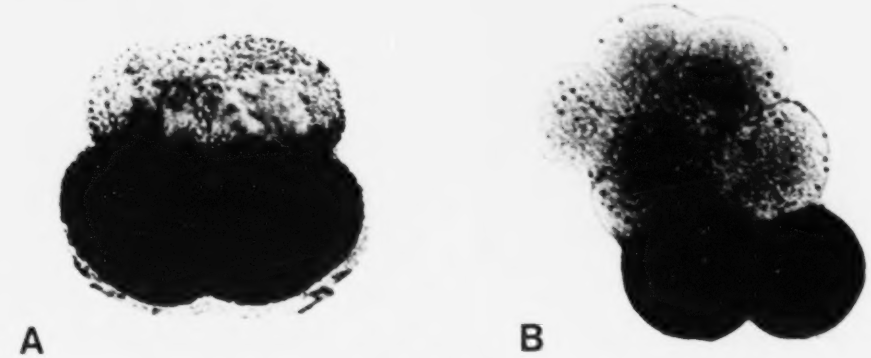

FIG. 4. Acetylcholinesterase activity in *Ciona* embryos cleavage-arrested with cytochalasin B until the time of normal hatching. A. 4-cell stage. B. 8-cell stage. ×370.

eage studies, two of these cells (the B4.1 pair) contain most of the potential for subsequent muscle development. We have confirmed this fate by isolating the B4.1 cell pair and studying the behavior of the developing partial embryo. Under the circumstances of our experiments it is indeed those cells which give rise to acetylcholinesterase-containing tissues (Whittaker *et al.*, 1977) and cells with myofibrils and myofilaments (Crowther and Whittaker, 1983) as illustrated in Figures 2D, 3B. Partial embryos from other isolated lineages do not develop these features (Figs. 2B, C).

The partial-"larvae" that result from B4.1 quarter-embryos exhibit only the differentiation markers, enzymatic and ultrastructural, associated with tissue and organ lineages still contained in the B4.1 cells (Crowther and Whittaker, 1983, 1986*b*; Whittaker, 1987). Hence, we are observing autonomy along with the restricted commitment predicted by the lineage diagram. This is further verification at a more refined level of the fact that early cleavage cells actually have become fixed in their fate. Isolation studies of cells taken from later division stages demonstrate that the capability of muscle expression follows the known muscle cell lineages (Meedel *et al.*, 1987).

An interesting additional method of demonstrating the autonomy of muscle cell development is the result of differentiation in cleavage-arrested embryos. When ascidian embryos were treated continuously with the microfilament assembly inhibitor cytochalasin B, they did not subsequently divide but remained fixed in the cleavage stage at which treatment began. Nuclear divisions appear to continue on schedule in cytochalasin-treated embryos (Crowther and Whittaker, 1986*a*), but further cytokinesis is prevented. Such embryos cleavage-arrested at various stages (2-, 4-, 8-cell, etc.) develop not only acetylcholinesterase in the muscle lineage blastomeres (and no others), but the muscle lineage blastomeres exclusively develop myofilaments and myofibrils (Whittaker, 1973; Crowther and Whittaker, 1983). Nishikata *et al.* (1987) have shown by immunocytochemistry that cleavage-arrested embryos also develop a myosin protein in their muscle lineage cells.

An example of acetylcholinesterase development in the two muscle lineage blastomeres of cleavage-arrested 4- and 8-cell stages is shown in Figure 4. This technique is interesting because it illustrates the same autonomy of expression observed in partial embryos resulting from isolated cells.

FUNCTIONS OF THE NUCLEUS

During the late 19th century there were a number of truly prophetic speculations about the control of development by interactions of the egg cytoplasm and the nucleus. Seen in the light of present understanding, many of these statements about the problem ring remarkably true. Among the more interesting and essentially correct discussions were those by Hugo de Vries (1889) and Hans Driesch (1894).

It was not, however, until the rediscovery of Mendelism by de Vries among others, and the subsequent relating of chromosomal inheritance mechanisms to Mendelian heredity by Boveri (1902) and Sutton (1903), that a proper basis for these speculations became known. Thereafter, conjectures about the possible role of "organ-forming substances" and egg cytoplasmic determinants in regulating the physiological functions of the nucleus took on a form that we would presently recognize. One of the best summary statements is still Wilson's (1925) classic treatise, *The Cell in Development and Heredity.*

Such a theory of developmental control was predicated on the understanding that division nuclei of an embryo remained essentially equivalent or pleuripotent. Again, a number of early observations and experiments testified to this likelihood, but only the results of nuclear transplantation studies in recent decades have resolved the question in a convincing manner (McKinnell, 1978). Tung *et al.* (1977) demonstrated by transplanting nuclei from organ regions of the older *Ciona intestinalis* embryo into nonnucleate egg fragments (cytoplasts) that the nuclei of ascidian embryos remain pleuripotent in their expression. In this respect, then, a highly "mosaic" embryo of the ascidian type is not different from the embryos of amphibians and fishes tested by earlier investigators.

Nuclear equivalence in a mosaic embryo is sufficiently important a point to merit replicating and extending the Tung experiments, which we did in collaboration with one of the original investigators, S. C. Wu (Wu, Crowther, and Whittaker, in preparation). Ultrastructural markers of histotype were used to verify the original conclusions about pleuripotency of nuclear function. In particular, we demonstrated in these transplant embryos the development of cytotypic features of nerve, muscle, notochord and epidermis, as we had defined them ultrastructurally in previous studies (Crowther and Whittaker, 1984, 1986*a*). We further extended the studies to cross-species nuclear transplantations between two distantly related species that do not ordinarily hybridize (*Styela clava* nuclei to *Ciona intestinalis* cytoplasts) and thereby found exceptional functional compatibility between nucleus and foreign cytoplasm.

The general theory about the selective expression of gene sets in embryonic development can be attributed to Morgan (1934, 1935). Simply stated, Morgan's idea was that egg cytoplasmic factors could function to *activate* specific genes at certain times of development. Among the more interesting later models concerned with explaining gene control in higher organisms are those of Davidson and Britten (1971) and Davidson (1986), which also entertain some possibility of differentially distributed gene activation factors. Our own ultrastructural observations of up to four kinds of histotypic differentiation features expressed simultaneously in cleavage-arrested ascidian zygotes suggest that determinants function autonomously and without interference in an undivided cytoplasm (Crowther and Whittaker, 1986*a*). Such findings are consistent with ideas of positively acting regulatory agents.

An unresolved question about the determination process is the extent to which some early chromosomal change might occur in response to cytoplasmic segregation of a preformed regulatory agent. The pleuripotency results of nuclear transplantation experiments suggest that any such

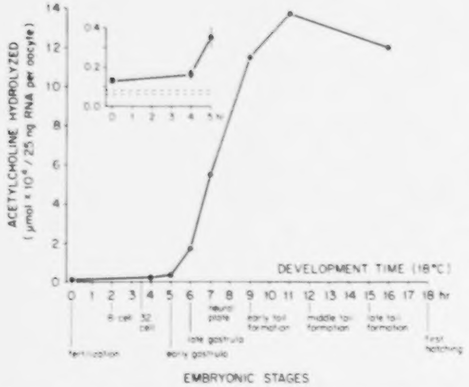

FIG. 5. Occurrence of translatable acetylcholinesterase mRNA in developing *Ciona* embryos. RNA was isolated from embryos at the stages indicated and injected into *Xenopus* oocytes. (From Meedel and Whittaker, 1984)

early change in a chromosome must lack a high degree of stability. If determination of the kind we observe in mosaic embryos is merely a later consequence of cytoplasmic agents segregated differentially during early cleavages, then there must be some critical time at which these "organ-forming substances" function. Presumably, then, there are mechanisms which release or activate these factors themselves at an appropriate later time. These considerations are part of the complex problem of explaining determinants and their function, as are questions about the exact cytoplasmic localization of the agents.

EXPRESSION OF THE ACETYLCHOLINESTERASE GENE

Purification of *Ciona* larval acetylcholinesterase by an affinity chromatography procedure and subsequent analysis of the enzyme by polyacrylamide gel electrophoresis revealed the ascidian enzyme to be composed of subunits of a single major polypeptide (Meedel, 1980). It seems likely, therefore, that only a single structural gene expression is involved in the development of this enzyme.

An antibody prepared to the purified enzyme (Meedel, 1980) formed the basis of a sensitive and specific method for measuring the relative amounts of acetylcholinesterase messenger RNA in purified whole RNA preparations from *Ciona* embryos of various ages. This technique follows the standard method by Gurdon (1974) whereby essentially linear translations of various mRNAs are obtained in the *Xenopus laevis* oocyte, after purified RNAs are microinjected into it. The enzyme protein resulting from translation of the ascidian acetylcholinesterase mRNA was assayed quantitatively by a radiometric assay of enzyme activity after precipitating it from oocyte homogenates with a combination of antibody and *Staphylococcus aureus* protein. Under carefully standardized conditions for oocytic translation and subsequent enzymatic assay of product, the amount of the enzyme found is proportional to the quantity of enzyme mRNA present (Meedel and Whittaker, 1983).

By this assay method, mRNA for *Ciona* larval muscle acetylcholinesterase was first found in RNA preparations taken from embryos at the early gastrula stage of development (Meedel and Whittaker, 1983, 1984). These results are shown in Figure 5. With a similar but less sensitive method (without antibody), Perry and Melton (1983) found the first translatable enzyme mRNA at the neural plate stage. These several studies show that no significant amount of translatable mRNA for acetylcholinesterase is present in the embryo before gastrulation. Presumably, then, the gene is not functioning before that time.

The results of earlier experiments in my laboratory (Whittaker, 1973; Meedel and Whittaker, 1979) and by others, from sequential treatment of ascidian embryos with the RNA synthesis inhibitor actinomycin D, indicated likewise that synthesis of new RNA was required for acetylcholinesterase development, also at about the time of gastrulation. On this basis, it seems unlikely that an inactive maternally derived mRNA for the enzyme occurs in the oocyte, to be processed at gastrulation into an active form. Isolation of the *Ciona* acetylcholinesterase gene, and subsequent preparation of a cDNA probe for identifying complementary sequences of the mRNA, will permit this question of timing of gene expression to be settled unequivocally. Work on this isolation is in progress.

Our quantitative measurements both of actinomycin D sensitivity (Meedel and Whittaker, 1979) and mRNA translation in the *Xenopus* oocyte (Meedel and Whittaker, 1983, 1984) indicate a relatively narrow time window during development when mRNA for *Ciona* larval muscle acetylcholinesterase is produced. That window is a 6-hour period from approximately 5 to 11 hr of development (at 18°C), or from gastrulation until just after the early larval tail formation stage (Fig. 5). Presumably, whatever localized and segregated cytoplasmic factor acts on acetylcholinesterase gene expression must act during this time. Possibly, quantity of the factor limits the extent of expression.

Cleavage-arrested early *Ciona* embryos which produced acetylcholinesterase at all, did so within the same quantitative range as did untreated embryos of a comparable age (Whittaker, 1983). They also produced enzyme at approximately the same time as in normal embryos. A similarly "normal" quantitative expression of enzyme occurred in partial embryos containing the muscle lineages (Meedel and Whittaker, 1984). Since cells in cleavage-arrested embryos have nuclear number and cytoplasmic volume relationships different from those found in normally developing muscle tissues, it appears that processes of genetic transcription and translation occur with exceptional temporal and quantitative fidelity. This finding has suggested also that egg cytoplasmic determinants may be rate limiting in their actions. Full enzyme expression in the partial embryos is consistent with the same conclusion.

EXPRESSION OF OTHER MUSCLE GENES

There is sufficient evidence from the effect of actinomycin D in preventing the development of ultrastructural features of ascidian muscle to suggest that most aspects of muscle differentiation are under direct gene control (Terakado, 1973; Crowther and Whittaker, 1984). As noted also from such studies of acetylcholinesterase development, there is a window effect with exposure to actinomycin D; treatment of later developmental stages with the drug does not suppress expression.

Meedel (1983) has studied quantitatively ascidian myosin ATPase activity and finds that its development follows a pattern similar to that of acetylcholinesterase. As noted previously with acetylcholinesterase, myosin ATPase had a restricted period of sensitivity to actinomycin D that was essentially over by the time enzyme activity was increasing linearly. In a recent study, Nishikata *et al.* (1987) obtained a monoclonal antibody to ascidian myosin heavy chain and have used this reagent at an immunocytochemical level to investigate muscle development. They also found a discrete actinomycin D sensitivity period.

Some interesting preliminary results have been described by Jeffery *et al.* (1986) using a cDNA probe to establish the occurrence of an ascidian maternal muscle actin mRNA. As yet there is no clear evidence that this actin mRNA is present in the zygote in sufficient amount to direct muscle actin synthesis during early development, whether it is even a functional mRNA *in vivo*, or if it is differentially segregated into muscle progenitor cells. For the moment, we are left with the conclusion on other grounds that ascidian muscle development probably begins exclusively through newly initiated gene expression during the early stages of development.

LINEAGE-RELATED GENE EXPRESSION

One would presume on the basis of various pieces of evidence from cell lineage phenomenology and actual phenotypic expression in isolated partial embryos that histospecific genes become activated only in their appropriate lineage cells. Our procedures for assaying the ascidian acetylcholinesterase mRNA in *Xenopus* oocytes allow us to answer this question directly. The results are consistent with our hypothesis that an egg cytoplasmic determinant is segregated into the muscle lineage cells. In turn, the agent presumably has some eventual relationship to the expression of muscle-specific genes in those cells.

Three kinds of partial embryo were prepared from *Ciona* 8-cell stages: an animal half-embryo obtained by isolating the animal quartet of cells, and two vegetal quarter-embryos prepared from the A4.1 and B4.1 cell pairs of the vegetal quartet (see Fig. 1B). Only the B4.1 cells contain the major or primary muscle lineages. After these partial embryos had developed to the time of "tail formation" stages, their RNA was isolated and tested for the presence of acetylcholinesterase mRNA (Meedel and Whittaker, 1984). Such RNA isolations could be done with as few as 25 operated embryos. It was only the B4.1 quarter-embryos which produced any indication of acetylcholinesterase mRNA formation, hence the acetylcholinesterase gene is shown to become active in the muscle lineages but not in tissues derived from the other lineages. Interestingly, the quantities of mRNA found were similar to those observed in normal embryos as part of the same experimental assays.

These findings eliminate from major consideration, at least in ascidian muscle development, models that suggest some gradient of gene expression or perhaps even a universal background expression of particular genes. In such circumstances, control is postulated to reside in the differential distribution of translational control factors rather than in agents which might regulate expression of the genes (Collier and McCarthy, 1981). Similarly, if there are gradients in the zygote of determinants for muscle and other tissues, as suggested by Catalano *et al.* (1979), one would not expect to find such an absence of acetylcholinesterase mRNA in nonmuscle lineage tissues. There may, however, be other individual and special circumstances where control at the translational level does govern primary developmental processes.

TRANSFER OF MUSCLE LINEAGE CYTOPLASM

Ultimate proof of the reality of cytoplasmic substances controlling lineage-specific histodifferentiations would be transferring histocapabilities between lineages by moving particular regions of egg cytoplasm. It has been possible, with respect to muscle, to accomplish such transfers and the resulting transformations successfully, but the more direct techniques of cytoplasmic microinjection have created anomalies which raise serious questions and problems. Presumably, one would wish to create a biological assay as a basis for testing the isolation and identification of one or more of the cytoplasmic determinants. At the moment there is still no practical assay available.

In retrospect, the first proof of the transfer of ascidian muscle determinacy was the centrifugation experiments of Conklin (1931). When he displaced myoplasm and other identifiable cytoplasmic regions centrifugally into different regions of the zygote, muscle and other tissues were accordingly displaced into those secondary regions in the abnormally developing embryo which resulted. For example, myoplasm (minus the excessive mitochondria which it ordinarily contained) seemed by itself to result in muscle differentiations in the regions of the embryo to which it was displaced centrifugally. These experimental results were not taken as seriously on the point of determinacy transfer as they perhaps should have been since they lacked selectivity and were in many respects rather interpretive, especially on the matter of tissue identifications at the histological level.

With the advent of the acetylcholinesterase marker of muscle differentiation that could be used simply at a histochemical level, experiments of a more selective nature were devised. The first of these was

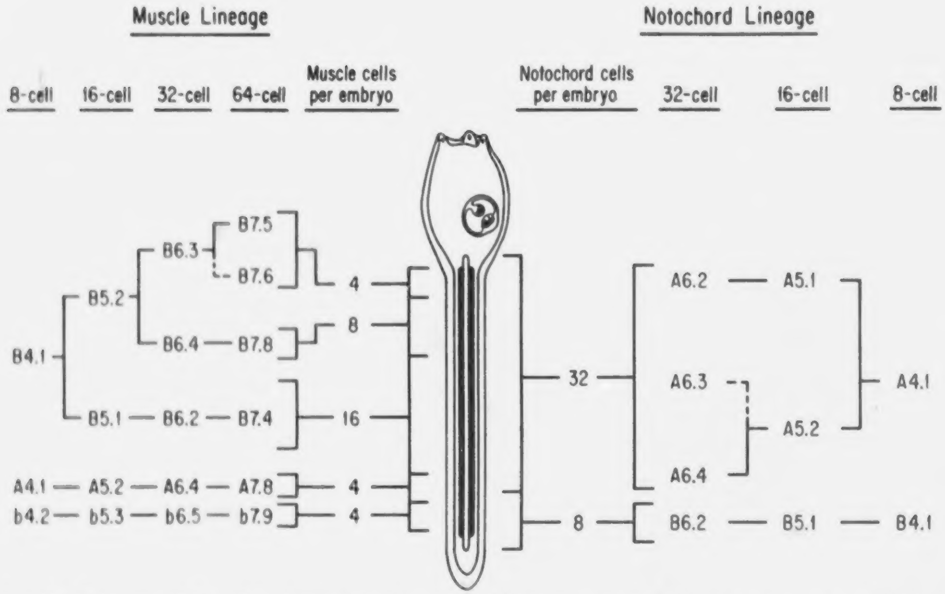
Muscle Lineage
Notochord Lineage
8-cell
16-cell
32-cell
64-cell
Muscle cells per embryo
Notochord cells per embryo
32-cell
16-cell
8-cell
B4.1
B5.2
B5.1
B6.3
B6.4
B6.2
B7.5
B7.6
B7.8
B7.4
4
8
16
A4.1 — A5.2 — A6.4 — A7.8
4
b4.2 — b5.3 — b6.5 — b7.9
4
32
8
A6.2 — A5.1
A6.3
A6.4
A5.2
A4.1
B6.2 — B5.1 — B4.1

FIG. 6. Fate map of *Ciona* larval muscle and notochord lineages as amended by the observations of Nishida and Satoh (1983, 1985). (From Meedel *et al.*, 1987)

a compression experiment done in the manner of Morgan, but using an ascidian species with a distinct yellow crescent myoplasmic region (*Styela plicata*). The 4-cell stage was compressed between two glass plates during third cleavage, causing the plane of third cleavage to be meridional rather than equatorial. The resulting 8-cell stage was a flat pie-shaped disc of cells which had visible segments of yellow crescent material in 4 cells instead of only in the two B4.1 cells of the normally cleaving embryo (Whittaker, 1980). When such embryos were cleavage-arrested at the 8-cell stage by continuous treatment with cytochalasin B, many of the later embryos had 3 and 4 blastomeres staining for acetylcholinesterase, instead of only the B4.1 pair in normal embryos cleavage-arrested at the 8-cell stage.

Another type of experiment involved "cutting" cytoplasm from the B4.1 muscle lineage cells into the b4.2 ectodermal lineage pair by shifting the cleavage plane from its normal position during "3rd cleavage." Posterior half-embryos were isolated at the 4-cell stage. As these two cells were dividing "equatorially" at next cleavage to form the animal b4.2 pair and the vegetal B4.1 duet, the cleavage furrow was shifted mechanically with a glass microneedle to include B4.1 cell cytoplasm in the newly enlarged b4.2 cell pair (Whittaker, 1982). About a third of these cytoplasmically enlarged b4.2 quarter-embryos developed patches of cells with acetylcholinesterase staining, similar to that shown in Figure 2D, but usually smaller.

Nishida and Satoh (1983) report from their cell lineage marking studies that b4.2 cells contain a secondary or minor muscle lineage contributing a few cells to the larval tail (Fig. 6). In our own examinations of several ascidian species, isolated b4.2 quarter-embryos do not autonomously differentiate acetylcholinesterase except when the animals from which the embryos were obtained had been subjected to extreme seasonal temperature stresses, nor do b4.2 quarter-embryos differentiate any traces of ultrastructurally identifiable muscle (Whittaker, 1982; Meedel *et al.*, 1987). The conclusion that transformation has occurred through the agency of cytoplasmic transfer would seem, therefore, to be valid in this particular experiment.

An interesting interpretation of the results from all three of the above experiments is that they indicate the possibility of positively acting factors being responsible for transformations to "muscle." If a major basis of the cytoplasmic determinant phenomenon is segregation of gene repressors or agents with otherwise negative effects *out of* a cell lineage at various early cleavages, one would not expect transformations to move directly *with* the transferred cytoplasm.

Deno and Satoh (1984) described the results of an experiment in which at the 8-cell stage they transferred portions of cytoplasm by microneedle from a B4.1 muscle lineage blastomere into an A4.1 blastomere. These injected 8-cell stages were then cleavage-arrested at the 8-cell stage with cytochalasin B until after the time of normal acetylcholinesterase development in that species (*Halocynthia roretzi*). Among their results, about 2% (8 embryos in 380 trials) were later found with acetylcholinesterase staining in an A4.1 blastomere. This is a problematical result for several reasons internal to the experiment.

Nishida and Satoh (1984) have shown (with *Halocynthia*) that A4.1 blastomeres contain some cells of a secondary muscle lineage. Indeed, they report elsewhere (Deno *et al.*, 1985) that 2% of *Halocynthia* quarter-embryos resulting from the isolation of A4.1 cell pairs differentiate acetylcholinesterase. It is noted by Deno and Satoh (1984) that none of 552 control embryos had acetylcholinesterase in extra blastomeres. Yet in experiments of our own with a species (*Ascidia ceratodes*) in which A4.1 quarter-embryos always developed acetylcholinesterase, cleavage-arrested 8-cell stages of this species had a very high percentage of embryos (53%) in which 1 or 2 of the A4.1 cells developed enzyme (Meedel *et al.*, 1987). Even in *Ciona*, where we also found, as did Deno *et al.* (1985), that A4.1 quarter-embryos produce no acetylcholinesterase, about 5% of cleavage-arrested 8-cell stages produce some light enzyme staining in a single A4.1 cell (Meedel *et al.*, 1987). Apparently inductive interactions do occur to a slight degree in cleavage-arrested embryos. It seems prudent, therefore, for several reasons to remain skeptical of the claim by Deno and Satoh (1984) to have devised a practical biological assay system for the muscle determinant.

Attempts of our own (Whittaker and Wu, in preparation) to cause "muscle" transformations by microinjecting cytoplasm from B4.1 cells to isolated a4.2 and b4.2 cell pairs were not successful. In two species studied (*Ciona* and *Ascidia*), needle injury alone resulted in activation of acetylcholinesterase expression in a few cells of many of the quarter-embryos so treated. In some circumstances, therefore, acetylcholinesterase expression behaves as an injury response protein. We have not yet found the conditions of experiment or embryo culture which will prevent this injury effect. In spite of its relative simplicity and convenience, caution must obviously be exercised in using acetylcholinesterase expression almost exclusively as a muscle differentiation character. Other muscle protein and gene probes will also have to be used in determinant response experiments.

INDUCTIVE INTERACTIONS AND DETERMINATION

The corrections to the ascidian lineage map introduced recently by Nishida and Satoh (1983, 1985) are especially interesting with respect to the location of the newly revealed cells in relation to those known originally to be part of the same tissues. As shown in the lower part of Figure 6, the terminal 8 of the 36 tail muscle cells of the larva come from lineages different from the initially identified B4.1. Similarly, the most distally located 8 of the 40 notochord cells are now known to originate in a different cell lineage from the A4.1, believed previously to contain all of the future notochord cells. Some of the difficulty with identifying all the lineage sources of the muscle and notochord cells was caused by the insensitivity of the techniques used to mark them; the techniques could not readily detect contributions from a minor source. Something considerably more, however, is involved. From their location, these newly identified cells have the appearance of having been recruited secondarily to lengthen the tail, and appear to make up populations different from what in evolution must have been the original tail cells.

We have looked with particular care at properties of the muscle cells originating from these so-called "secondary" lineages (Meedel *et al.*, 1987). In *Ciona* there is no autonomy of expression in the secondary muscle cells: A4.1 and b4.2 quarter-embryos develop neither acetylcholinesterase nor ultrastructural features of muscle (Fig. 2). Cell recombination experiments indicate for the A4.1-derived cells that associations with other embryo cells are required for their muscle expression. Also, the secondary lineage cells produce their acetylcholinesterase at a significantly later time, by several hours, than do the proximally located majority of B4.1-derived muscle cells. For technical reasons, it is less clear which other tissues the b4.2-derived cells must interact with to achieve muscle differentiation and what their timing relationships might be, if different from the A4.1 lineage cells.

One might judge from these findings that the determinative properties of the secondary muscle lineages are different from the majority derived from the B4.1 quarter-embryos, where there is clearly strong autonomous development of muscle characteristics. This absence of "self-differentiation" lends support to the idea of a separate origin of the secondary muscle lineages that may not involve segregation of the same, or indeed, of any cytoplasmic determinant. The findings of probable inductive interactions also serve once again to emphasize the dual nature of determinative controls mentioned at the beginning of this review.

CONCLUSIONS

The evidence presented here leaves little doubt that egg cytoplasmic agents are involved in the determination of ascidian muscle development. Their actual function is uncertain. Much of the evidence is consistent with a possibility that the muscle factor is a positively acting link in the chain of regulatory events which ultimately initiate muscle gene activity. Acetylcholinesterase factor is not a preformed maternal mRNA for the enzyme; enzyme development follows from new gene activity. Since there is significant new mRNA synthesis during ascidian development it seems likely that most of larval development proceeds from new gene activity during embryogenesis (Meedel and Whittaker, 1978). Cytoplasmic determinants remain, therefore, candidates for possible gene control elements.

Yet there is one clear example of a strong maternal effect during larval development involving expression of a histospecific endodermal alkaline phosphatase. Autonomous expression of the alkaline phosphatase in partial embryos containing endodermal lineages, and enzyme development in endodermal lineage cells of cleavage-arrested embryos, both indicate a very early commitment to its later expression. Formation of the enzyme is resistant to treatment with actinomycin D (Whittaker, 1977, 1987; Bates and Jeffery, 1987) and seems thereby to result from a masked maternal mRNA. Nonetheless, enzyme fails to develop in activated anucleate egg fragments (Bates and Jeffery, 1987), which indicates some essential function of the nucleus in the eventual release of expression. Insensitivity of alkaline phosphatase gene expression to actinomycin D cannot yet be excluded as a possibility.

Where the determinants are located within the zygote and later embryonic cells is now only beginning to be addressed. The muscle determinant seems to reside in a cytostructural myoplasmic domain associated with the mitochondrial crescent and which can be isolated by mild detergent extraction (Jeffery, 1985). If, as seems likely, determinants are anchored in specific regions of the cytostructure and thereby become segregated appropriately, then how they eventually leave the cytostructure and become functionally active at an appropriate developmental stage are important events for which no likely mechanisms have been suggested.

Ultimately, we must confront the problem of the origin of the cytoplasmic determinants. Certainly they result from gene activities during oogenesis, but how the determinants become temporally and spatially ordered in the oocyte is not presently investigatable. Learning the chemical identity of one or more of the determinants is an important first step in devising a research strategy. An extensive rearrangement of egg cytoplasmic regions occurs immediately after fertilization (Conklin, 1905*a*; Ortolani, 1955) and various investigators have now begun to address the nature of these changes by employing modern investigative techniques (Sawada, 1983). One hopes that an understanding of the mechanics of zygotic reorganization will also offer some insight into possible manipulative techniques for exploring determinant formation during oogenesis.

ACKNOWLEDGMENTS

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Genomic Potential of Differentiated Cells Analyzed by Nuclear Transplantation[1]

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SYNOPSIS. The theory of nuclear equivalence proposes that specialized somatic cells of metazoans possess a gene pool identical to that present in the zygote nucleus. We examine this theory on the basis of nuclear transplantation experiments in amphibian oocytes and eggs. This procedure has the potential to test the entire genome and to evaluate the problem of nuclear equivalence in the context of a functioning organism.

Nuclear transplantations from several differentiated somatic cell types into oocytes and eggs have revealed that their nuclei still contain the genes required for the development of prefeeding tadpoles. In addition, erythrocyte nuclei have directed the formation of feeding tadpoles that advanced to stages of hind limb bud. Thus, the genome of several differentiated somatic cells displays genetic multipotentiality. Although evidence for the genetic totipotency of specialized somatic cells is lacking, the results of our recent experiments suggest that the genetic totipotency of at least some differentiated somatic cell types still remains a tenable hypothesis.

INTRODUCTION

During the late 19th century scientists introduced experimental approaches to examine the genomic potential of differentiated cells, the subject of this review. They sought to determine whether or not the somatic nuclei of the diverse specialized cells of adults remained equivalent to the zygote nucleus. During this period Weismann (1892) proposed a theory of genetic differences among the progeny of the zygote nucleus to account for the diversity and stability of specialized somatic cell types. His model stated that the immediate progeny of the zygote nucleus lost genetic determinants, and that this loss continued in a progressive and specific manner, such that, *e.g.*, liver cells contained a repertoire of determinants required for liver cells, while each of the other somatic cell types possessed a different set of determinants. Only the germ cells were exempt from genetic loss. Thus, Weismann proposed an irreversible mechanism for somatic cell specialization. However, this proposal was refuted for early cleavage nuclei in several invertebrate and vertebrate species. For example, sea urchin embryos during early cleavage were subjected to pressure between glass plates, thus displacing the nuclei so that they were situated in different regions of the embryo. Nevertheless, normal development ensued when the compression was released (Driesch, 1892*a*). Separation of sea urchin blastomeres at the two-cell stage resulted in two whole embryos, but dwarfed (Driesch, 1892*b*). Similarly, frog (McClendon, 1910) and salamander (Ruud, 1925) blastomeres separated at the two-cell stage could each produce normal but small embryos. Delayed nucleation experiments in eggs of the sea urchin (Loeb, 1894), salamander (Spemann, 1914), and dragonfly (Seidel, 1932) provided evidence for the equipotentiality of cleavage nuclei for at least the 16–128 cell stage. These examples cited as well as other studies demonstrated nuclear equivalence during early cleavage stages in many species. However, deletions or redistributions in cytoplasmic regions of mosaic eggs interfered with normal development. Thus, the egg was not composed of homogeneous cytoplasmic constituents, and this was most clearly demonstrated in the mosaic eggs, where specific cytoplasmic inclusions control the initial determination of embryonic regions (reviewed in Morgan, 1927; Wilson, 1928; Spemann, 1938; Briggs and King, 1959; McKinnell, 1978; Moore, 1987).

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These initial studies left unresolved the question of nuclear equivalence for later stages. Do the nuclei remain identical and totipotent or do they change in response to the different cytoplasmic environments? Hans Spemann (1938) in his Silliman Lectures at Yale University discussed this problem:

> From the assumption of Weismann concerning the differential division of the nucleus, there would follow immediately a restriction of the potency of the genome; for if the germ plasm were separated, during development, into its constituent parts and distributed over the single cells of the body, it is evident that each of these cells would contain only its allotted portion and would lack all the remainder. But by refuting of this fundamental hypothesis one cannot conclude that each cell of the body will contain the whole undiminished idioplasm; for genes may be lost or become ineffective in other ways besides that of elimination out of the cell. Decisive information about this question may perhaps be afforded by an experiment which appears, at first sight, to be somewhat fantastical. It has been shown, as pointed out before, in the egg of the sea urchin (Loeb, 1894) and the newt (Spemann, 1914) that a piece of the egg protoplasm which contains no nucleus may be induced to develop, may be "fertilized," as it were by a descendant of the fertilized egg nucleus. In this experiment the nucleus to be tested is transmitted through a bridge of protoplasm from the one half of the egg in which it had originated into the other half which had hitherto been devoid of a nucleus. Probably the same effect could be attained if one could isolate the nuclei of the morula and introduce one of them into an egg nucleus. The first half of this experiment, to provide an isolated nucleus, might be attained by grinding the cells between two slides, whereas for the second, the introduction of an isolated nucleus into the protoplasm of an egg devoid of a nucleus, I see no way for the moment. If it were found, the experiment would have to be extended so that older nuclei of various cells could be used. This experiment might possibly show that even nuclei of differentiated cells can initiate normal development in the egg protoplasm. Therefore, though it seems an anticipation of exact knowledge to say that 'every single cell possesses the whole apparatus of potencies' (Petersen, 1922, p. 116), yet this opinion may be right.

As we shall see below, Spemann's prophecy has been upheld to this extent: nuclei of differentiated somatic cells have *initiated* apparently normal development of early amphibian embryos, but so far no nucleus of a differentiated somatic cell has led to the formation of a normal frog. In the jargon of developmental biologists, "genetic totipotency" has not yet been shown for nuclei of specialized somatic cells. What follows is a review of the implementation of Spemann's proposal of the "fantastical experiment." We will show that after a long and arduous road of experimental work, evidence has now accumulated demonstrating that some specialized somatic nuclei are genetically multipotential. We shall also see that Spemann's proposal, conceived about fifty years ago, was elegant in its day, but was conceptually oversimplified. It now appears that nuclei from both somatic and germinal specialized cells pose special requirements that cannot be totally satisfied in the egg. The task of answering the question of the genomic potential of differentiated cells is now left to current and future scientists.

NUCLEAR TRANSPLANTATION

Blastula and early gastrula nuclei

Spemann died in 1941, but just eleven years later, Briggs and King (1952) accomplished nuclear transplantation in amphibian eggs, a procedure that tests the genetic potencies of metazoan nuclei. In their initial studies they used frog nuclei from the animal hemisphere cells of blastula and early gastrula embryos, stages that consisted of 8,000 to 16,000 cells. The nuclei of these cells had traversed many more cell cycles and had been exposed to embryonic cytoplasm for a much longer time than had the early cleavage nuclei referred to previously. The nuclear transplantation procedure, initially developed for the leopard frog *Rana pipiens*, involves three main steps: activation of the egg, microsurgical removal of the egg nucleus to produce an enucleated host, and transplantation of a test nucleus with its disrupted cell components into the animal hemisphere region of the previously prepared activated and enucleated egg (Briggs and King, 1952; King, 1966, 1967; DiBerardino *et al.*, 1977; McKinnell, 1978). The type of development that follows is a reflection of the genetic potential of the test nucleus. These studies revealed that many blastula and early gastrula nuclei promoted development of the enucleated eggs into normal larvae (Briggs and King, 1952), that metamorphosed into juvenile frogs (Briggs and King, 1960). In 1958 Fischberg and collaborators initiated nuclear transplantation studies in the South African frog *Xenopus laevis*, and also obtained normal tadpoles from nuclei of early embryonic stages. In 1962 McKinnell and Gurdon independently obtained fertile frogs and provided the first evidence for genetic totipotency in nuclear transplantation experiments. These frogs were derived from blastula nuclei in *Rana* (McKinnell, 1962) and from nuclei of various stages in *Xenopus* (blastula to hatched tadpole; Gurdon, 1962*a*). Totipotency of amphibian embryonic nuclei was confirmed in the newts, *Axolotl* and *Pleurodeles* (see Signoret, 1965), and extended to the insect *Drosophila* (Illmensee, 1973; Zalokar, 1973) and to the teleost fish (Yan *et al.*, 1984).

More recently, nuclear transplantation was extended to mammals. Fertile mice, derived from enucleated zygotes transplanted with nuclei from the inner cell mass of the blastocyst, were reported by Illmensee and Hoppe (1981). McGrath and Solter (1983) obtained fertile mice only when pronuclei of the one-cell stage were fused as pronuclear karyoplasts to enucleated zygotes, but nuclei from subsequent preimplantation stages failed to support development beyond the blastocyst stage (McGrath and Solter, 1984*a*). However, Surani *et al.* (1986) were successful in obtaining full term mice from nuclei of early cleavage stages. They fused a haploid maternal nucleus from two-to-sixteen cell parthenogenetic or gynogenetic embryos back to a fertilized egg from which the female pronucleus was first removed. Some of these reconstituted eggs developed normally and reached term. Also, a haploid paternal nucleus from two-to-four cell androgenetic embryos was fused to a fertilized egg from which the male pronucleus had been removed. Some of these reconstituted eggs developed normally to term and both male and female progeny were obtained. Since mouse blastomeres of the eight-cell stage have been shown by recombination of blastomeres to be totipotent (Kelly, 1979), one would expect successful nuclear transplantation up to at least the eight-cell stage of the mouse when all the technical parameters are satisfied. Recently, three full term lambs were produced from single blastomeres from 8-cell sheep embryos, when the blastomeres were fused to enucleated halves of unfertilized eggs (Willadsen, 1986).

Advanced embryonic nuclei

Once the answer was obtained that amphibian nuclei of blastulae and early gastrulae were totipotent, investigators addressed the question of whether nuclei remained equivalent in later stages of embryogenesis. Nuclear transplantations of various types of cells from different embryonic stages, germ layers and primitive organs from various anuran and urodelen amphibians (*Ambystoma, Bufo, Pleurodeles, Rana,* and *Xenopus*) revealed a progressive decrease in the percentage of normal nuclear transplants from donor nuclei of older stages of embryogenesis (reviewed by: Briggs and King, 1959; Gallien, 1966; King, 1966; DiBerardino and Hoffner, 1970; Gurdon, 1974, 1986; McKinnell, 1978; Briggs, 1979). Other main points from these early studies were the following: (1) a minority of nuclei still displayed genetic totipotency, and this was most clearly demonstrated in the endodermal nuclei of hatched larvae of *Xenopus* (Gurdon, 1962*a*); (2) but the majority of nuclei did not promote normal development of the test eggs; (3) the developmental restrictions analyzed in endodermal (King and Briggs, 1956; Gurdon, 1960*a*; Subtelny, 1965) and neural nuclear transplants (DiBerardino and King, 1967) were stable, since they could not be reversed through serial transplantation; (4) furthermore, the restrictions expressed by endodermal nuclei were not corrected by parabiosing nuclear transplant embryos with normal ones (Briggs *et al.*, 1961), nor could they be corrected when a haploid set of egg chromosomes was combined with the diploid set from the endodermal nucleus (Subtelny, 1965).

Chromosome studies

One possible explanation for the nuclear restrictions displayed by the majority of nuclei from advanced cell types is that irreversible genetic changes do accompany embryogenesis, and that these occur at a later time than that proposed by Weismann (1892). Another possibility is that the chromosomal cycle of the test nucleus fails to integrate with the cytoplasmic division cycles of the recipient egg. Such an asynchrony could lead to chromosome abnormalities. I explored this latter possibility by analyzing the chromosomes of *Rana* nuclear transplants and some of our findings were confirmed in other species (King and Briggs, 1956; reviewed by DiBerardino, 1979). The main conclusions drawn from these studies were the following: (1) most abnormal nuclear transplants possessed detectable abnormalities in chromosome number and/or structure; (2) there was a correlation between the extent of development and the severity of the chromosome aberrations; (3) there was a progressive increase in the percentage of nuclear transplants with chromosomal aberrations from donors of later stages of development; and (4) some of the abnormal nuclear transplants were karyotypic mosaics, *i.e.*, a single nuclear transplant consisted of cells with the apparent normal karyotype, while other cells in the same individual exhibited various abnormal karyotypes. This latter observation was particularly informative, for it suggested that the original genome had not been damaged in the transplantation procedure, and that abnormal replicates of the donor genome were synthesized in some cells. In addition, the existence of karyotypic mosaicism provided an explanation for why subcloning of blastula nuclear transplants from advanced cell types could result in better development (DiBerardino and King, 1965; DiBerardino, 1979).

Studies of endodermal nuclear transplants within the first few hours following nuclear transplantation revealed that the chromosome abnormalities were associated with an asynchrony between the cytogenetic events of the transplanted nucleus and the recipient egg (DiBerardino and Hoffner, 1970). In the majority of cases the chromatin decondensation–condensation cycle of the transplanted nucleus was delayed, leading to abnormal condensation patterns in the metaphase chromosomes of the first mitotic cycle, followed by chromatid bridges at anaphase. Subsequent cleavage stages then exhibited aneuploidy and/or structural alterations in the chromosomes. The results of the chromosome studies on abnormal nuclear transplants revealed that the extensive chromosome aberrations were sufficient to account for the abnormal development of the nuclear transplants as well as their developmental arrest.

A series of studies has demonstrated that the state of the cytoplasm controls chromosome condensation, DNA synthesis and mitosis. Over sixty years ago, A. Brachet (1922) observed that when sperm prematurely entered oocytes of the sea urchin, which were still undergoing maturation divisions, the sperm chromatin rapidly condensed into chromosomes similar to those of the oocyte. The same phenomenon was later reported in precociously inseminated oocytes of amphibians (Bataillon and Tchou-Su, 1934). Since these classical studies were reported, the cytoplasmic control over nuclear activities has been extensively studied in amphibian eggs and oocytes by nuclear transplantation and in cultured cells by cell fusion. Donor nuclei injected into mature activated eggs behave like pronuclei; they enlarge, undergo chromatin decondensation (Subtelny and Bradt, 1963; Graham *et al.*, 1966) and synthesize DNA (Graham *et al.*, 1966). During the diplotene stage of the oocyte when the resident nucleus (germinal vesicle) is greatly enlarged, transplanted embryonic (Subtelny, 1968) and brain (Gurdon, 1968) nuclei also enlarge. After germinal vesicle breakdown when the oocyte's own chromosomes condense and become aligned on the spindle of the first meiotic metaphase, transplanted embryonic (Subtelny, 1968; Aimar and Delarue, 1980), brain (Ziegler and Masui, 1973; Brun, 1974) and erythrocyte nuclei (Aimar and Delarue, 1980; Leonard *et al.*, 1982) also transform into metaphase chromosomes on spindles. The same phenomenon has been observed in cultured mammalian cells when cells from different phases of the cell cycle are fused together. Cells in the G_1 phase can be induced to undergo DNA synthesis when fused with cells in the S phase, whereas G_2 cells fused with S cells cause the S cell nuclei to enter mitosis sooner, and mitotic cells fused with G_1, G_2 or S cells cause premature chromosome condensation (Johnson and Rao, 1970; Rao and Johnson, 1970; Matsui *et al.*, 1972).

The evidence of asynchrony between the transplanted nucleus and the cell cycle of the egg did not resolve whether or not the genome of advanced cell types had undergone irreversible genetic changes prior to nuclear transplantation. However, it suggested that a better coordination of cytogenetic events might lead to enhanced expression of genetic potential (DiBerardino and Hoffner, 1970).

Differentiated somatic tissues

Recall that the critical experiment posed by Spemann concerned the genetic repertoire of differentiated somatic cells. Subsequent to the studies on nuclei from advanced embryonic stages, the main interest in our field has centered on the developmental capacity of somatic nuclei from larvae and adults because their tissues consist of a large proportion of cells that have attained a high degree of specialized functions. This review emphasizes nuclear transplantation of differentiated cells, because they provide a critical test for the theory of nuclear equivalence. Extensive reviews of nuclear transplantation experiments on embryonic nuclei have been published (Briggs and King, 1959; Gallien, 1966; King, 1966; DiBerardino and Hoffner, 1970; Gurdon, 1974, 1986; McKinnell, 1978; Briggs, 1979).

Nuclear transfer experiments that assess the genetic potential of differentiated cells must include evidence for two conditions: (1) the donor cells are differentiated, and (2) the nuclear transplants are derived from the donor nucleus, and not from the egg nucleus. In order to fulfill these conditions, the donor cells must be unequivocally identified by direct observation under the microscope at the time of transplantation to permit direct documentation of their differentiated state. When visual identification of the donor cells is not possible, it is necessary to determine that the percentage of undifferentiated cells in the donor population or in an equivalent population is so small that the percentage of nuclear transplants that develops exceeds significantly the percentage of undifferentiated cells in the population. Verification that the nuclear transplants developed from the transplanted test nucleus and not from an egg nucleus that remains because of faulty enucleation has been obtained in the following ways. In *Xenopus* the egg nucleus is inactivated by UV irradiation (Gurdon, 1960*b*), but since this procedure is successful in only 61–92% of the cases (Du Pasquier and Wabl, 1977), a genetic marker is required. The most used marker in *Xenopus* has been the 1-nu nucleolar mutation (Elsdale *et al.*, 1960) that can be seen in cells from gastrula stage and beyond. However, as pointed out by Du Pasquier and Wabl (1977), it is necessary to verify that one nucleolus is accompanied by a diploid set of chromosomes and does not result from haploidy or aneuploidy. Another genetic marker, available in *Xenopus* but so far less used, is the a^p albino mutant evident in the young swimming tadpole and beyond (Hoperskaya, 1975). In *Rana pipiens*, triploidy has served as a convenient cytogenetic marker (Gallien *et al.*, 1963; McKinnell *et al.*, 1969; DiBerardino *et al.*, 1983, 1986; Orr *et al.*, 1986), because the triploid set of chromosomes can be identified throughout the life cycle of the frog. For nuclear transplants that develop to late larval stages and beyond the *kandiyohi* (McKinnell, 1962) and *burnsi* (Simpson and McKinnell, 1964) pigment mutants of *Rana pipiens* are excellent genetic markers. Other naturally occurring pigment mutations have been summarized by McKinnell (1978) and could be used for genetic markers. Another type of verification procedure has also been used in *Rana pipiens* because microsurgical removal of the egg nucleus can be accomplished in 98–100% of the cases. The exovate formed at the time of enucleation adheres to the vitelline membrane outside of the egg. This membrane is later removed, sectioned and stained with the Feulgen procedure that specifically stains DNA and reveals the presence of the egg nucleus in the exovate. This information together with the determination of chromosome number of the nuclear transplant and the time of first cleavage insures that development ensued from the test nucleus (DiBerardino and Hoffner, 1971, 1983; Orr *et al.*, 1986).

We shall consider two series of experiments which differ in the certainty that donor cells were derived from differentiated somatic tissues. The first series (Table 1) comprises the initial attempts to work with differentiated cells at a time when the techniques usually precluded distinction beteen undifferentiated stem cells and differentiated cells in the tissues employed. In the second series (Table 2) the specialized properties of the donor cells were documented. In both series only those nuclear transplants that developed to postneurula stages, larval stages and adults will be considered. Postneurula embryos have developed primitive organ systems, and in the latter stages, display muscle and heart function. During the early larval stages the major cell types, tissue and organ systems have differentiated and are functional. Thus, transplanted nuclei capable of programming eggs to develop to these stages are considered to be genetically multipotent. Nuclei that direct the formation of fertile frogs are interpreted to be totipotent.

Nuclear transfers from differentiated somatic tissues

The advanced development of nuclear transplants obtained from differentiated somatic tissues of *Xenopus* larvae and adults is summarized in Table 1. Five adult frogs were obtained from 5 original larval nuclei, 4 from nuclei of the larval intestine (Gurdon, 1962*b*; Gurdon and Uehlinger, 1966), and 1 from a nucleus of larval epidermis (Kobel *et al.*, 1973). Three of these adult frogs were fertile, 2 from intestinal nuclei, and 1 from a non-ciliated cell of the epidermis. The donor cells from the intestine were selected on the basis of size. The epidermal cell from the experiments of Kobel *et al.* was identified as nonmotile (non-ciliated). Nuclei from epidermal cells that were motile (ciliated) and therefore specialized did not give advanced development. The authors concluded that the single case showing totipotency was derived from a non-specialized cell.

In addition to the studies tabulated in Table 1, endodermal nuclei of the primitive gut taken from postneurula embryos and initial larval stages before tissue differentiation directed *Xenopus* eggs to develop into 20 fertile frogs (~3%), the majority of which bore the 1-nu genetic marker of the donor nucleus (Gurdon, 1962*a*, 1986).

At least 4 metamorphosed frogs developed from eggs injected with larval nuclei (Table 1), one from an intestinal cell selected on the basis of size (Marshall and Dixon, 1977), and 3 from cell cultures (Gurdon and Laskey, 1970). The latter 3 were obtained from cell cultures of whole tadpoles, displaying the beginning of circulation in the gills, but it is not certain that the donors came from differentiated cells. The above named tissues (intestine, epidermis and cell cultures of tadpoles) also contained nuclei capable of directing eggs to develop into 69 postneurula embryos and 50 of these proceeded into larval stages.

Nuclear transfers of adult somatic nuclei into *Xenopus* egg resulted in 38 postneurula embryos and 15 of these developed into larvae. The somatic nuclei were derived from a cell line established from liver (Kobel *et al.*, 1973), from cell cultures of skin, lung, and kidney (Laskey and Gur-

TABLE 1. *Nuclear transfers from differentiated tissues.*

Donor		Identification of donor cells	Total # nuclei tested	No. (%) total transfers[a] reaching stage of				Verification	Reference
Stage	Tissue			Post-neurula	Larva	Meta-morphosis	Adult		
Larva	Intestine[X]	size	726	36 (5.0)	31 (4.3)		4[2F] (0.6)	1-nu	Gurdon, 1962*b*; Gurdon and Uehlinger, 1966
	Intestine[X]	size	522	8 (2.0)	8 (2.0)	1 (0.2)		98% haploids[b]	Marshall and Dixon, 1977
	Epidermis[X]	non-ciliated	440	2 (0.4)	2 (0.4)		1[F] (0.2)	1-nu	Kobel *et al.*, 1973
	Minced tadpoles[X]	cell culture	3,686	23 (0.6)	9 (0.2)	3 (0.1)		1-nu[c]	Gurdon and Laskey, 1970
Adult	Liver[X]	cell line	365	2 (0.6)	2 (0.6)			—	Kobel *et al.*, 1973
	Skin, lung, kidney[X]	cell culture	2,322	26 (1.1)	7 (0.3)			1-nu[c]	Laskey and Gurdon, 1970
	Intestine[X]	size	1,112	10 (0.9)	6 (0.5)			95% haploids[b]	McAvoy and Dixon, 1975
	Testis[R]	size	116	4 (3.5)	1 (0.9)			exovate[c]	DiBerardino and Hoffner, 1971

X, *Xenopus laevis*; **R**, *Rana pipiens*; **F**, fertile; 1-nu, one nucleolus.

[a] Includes the results of serial transfers.

[b] Denotes efficiency of UV irradiation in controls.

[c] Chromosome number determined in nuclear transplants.

TABLE 2. *Nuclear transfers from differentiated cells.*

Donor		Differentiated state of donor cells	Total # nuclei tested	No. (%) total transfers[a] reaching stage of				Verification	Reference
						Feeding larva			
Stage	Tissue, cell			Post-neurula	Larva	Hindlimb bud	>		
Embryo	Myotome[X]	shape	176	4 (2.0)	4 (2.0)			1-nu[b] a^p	Gurdon *et al.*, 1984
Larva	Gonads, primordial germ cells[R]	size	410	35 (8.5)	31 (7.6)		10[c] (2.4)	99.5% haploids[d]	Smith, 1965
	Melanophores, cell culture[X]	pigmented	257	2 (0.8)				1-nu	Kobel *et al.*, 1973
Juvenile frog	Erythrocytes[R]	shape, hemoglobin	51	4 (8.0)	4 (8.0)	3 (5.9)		triploid[b]	DiBerardino *et al.*, 1986
Adult	Erythrocytes[R]	shape, hemoglobin	130	11 (8.5)	6 (4.6)			exovate[b]	DiBerardino and Hoffner, 1983
	Erythroblasts[X]	shape, hemoglobin	442	8 (2.0)	8 (2.0)			1-nu[b]	Brun, 1978
	Skin, cell culture[X]	keratin antibody	129	6 (4.6)	4 (3.1)			1-nu[b]	Gurdon *et al.*, 1975
	Spleen[X]	immunogen antibody	100	6 (6.0)	6 (6.0)			1-nu[b]	Wabl *et al.*, 1975

X, *Xenopus laevis*; R, *Rana pipiens*; 1-nu, one nucleolus; a^p, albino mutant.

[a] Includes the results of serial transfers.

[b] Chromosome number determined in nuclear transplants.

[c] Ten larvae raised, final stage not indicated, see text.

[d] Denotes efficiency in microsurgical removal of the egg nucleus.

don, 1970) and from *in vivo* intestinal cells selected on the basis of size (McAvoy and Dixon, 1975).

The conclusions that can be drawn from the above studies are the following. First, cells from both larval and adult differentiated tissues contain some nuclei that can direct the formation of the diverse cell types found in postneurula embryos and tadpoles. Therefore, these nuclei display genetic multipotentiality. Second, genetic totipotency has been demonstrated for some advanced *embryonic* and young *larval* nuclei, since these nuclei directed the formation of fertile frogs. Third, the interpretation of these results remains equivocal, because with the exception of the nonciliated epidermal cells, no relevant criteria were used to distinguish between undifferentiated stem cells and differentiated cells in the tissues employed.

Nuclear transfers from differentiated somatic cells

In this section we shall consider the advanced development obtained from somatic nuclei of donor cells that unequivocally displayed a differentiated state. Among the cell types listed in Table 2, elongated cells of the embryonic myotome (Gurdon *et al.*, 1984), pigmented larval melanophores (Kobel *et al.*, 1973), and oval erythroid cells (Brun, 1978; DiBerardino and Hoffner, 1983; DiBerardino *et al.*, 1986) were directly identified by their appearance preceding injection. Erythroid cells are particularly easy to identify because of their red color due to their hemoglobin. In the case of the skin (Gurdon *et al.*, 1975) and spleen (Wabl *et al.*, 1975) studies, the donor cells were derived from a nearly homogeneous population. Evidence that the donor cells had synthesized a specialized molecular product was provided in the following ways. Correlative studies of myotome tissue showed that somite cells were making mRNA for muscle specific actin, and correlative studies of cultured skin cells revealed that over 99.9% of the cells reacted with a keratin antibody. In the case of the spleen cells, 96.1 to 98.7% of the donor cells were judged to be immunoglobulin producing cells (B lymphocytes) because the population of donor cells bound to a specific antigen. Finally, the pigment in the melanophores and the hemoglobin in the erythroid cells provided direct evidence of their unique specialized products. Nuclei from all of these somatic cell types promoted the formation of a total of 41 postneurula embryos. With the exception of cultured melanophores, nuclei from all the other cell types promoted the formation of 32 larvae. Thus, there is evidence for the genetic multipotentiality of nuclei from specialized cells of the advanced embryo, larva, juvenile frog and adult. *However, to date, no nucleus of a documented specialized cell nor of an adult cell has yet been shown to be totipotent.*

The erythrocyte nuclei from *Rana pipiens* yielded the highest percentage of postneurula embryos (8.5%; DiBerardino and Hoffner, 1983) and swimming tadpoles (8.0%; DiBerardino *et al.*, 1986). Furthermore, 5.9% of the erythrocyte nuclei from juvenile frogs supported the formation of feeding tadpoles that survived up to a month and formed hind limb buds (DiBerardino *et al.*, 1986). This was an important finding because the other tadpoles derived from specialized cells developed in the best cases up to the feeding stage, but failed to feed and to survive. The main difference in the experimental procedure was that the erythrocyte nuclei were initially injected into oocytes, whereas the other nuclear types were injected directly into eggs. We shall elaborate on this recent modification of the procedure in a later section.

Germ cell nuclei

Nuclear transplantation tests of germ cell nuclei are of special interest, because the complete male or female genome is present in germ cells. Unfortunately, there have been only two studies that examined the developmental potential of germ cells. First, Smith (1965) transplanted nuclei of primordial germ cells taken from *Rana pipiens* tadpoles at the initial feeding stage (Table 2). These cells are large and yolky and can be easily distinguished from the small somatic cells of the gonads. Normal tadpoles (31) developed from 7.6% of the injected eggs. Ten of these tadpoles were reared for periods of one to three months, were then autopsied and found to be normal. Although the exact larval stages are not reported, except for a photograph of a metamorphosing tadpole, these tadpoles have developed further than those from documented differentiated somatic cells.

The other study concerned nuclei from the testes of adult *Rana pipiens* (DiBerardino and Hoffner, 1971). The cell suspension of donor cells was enriched for spermatogonia and the donor cells were selected by diameter measurements with an ocular micrometer. Enucleated eggs injected with these nuclei yielded 4 postneurulae and 1 larva (Table 1). Although complete normal development did not ensue from these differentiated germ cell nuclei, the tadpole fed and was consideraby more differentiated than those obtained from the somatic nuclei of the documented specialized cells summarized above, with the exception of the feeding tadpoles recently derived from erythrocyte nuclei of juvenile frogs (DiBerardino *et al.*, 1986).

NUCLEAR TRANSFER IN MEIOTIC OOCYTES

Rationale and procedure

Although genetic multipotentiality was shown for some somatic nuclei of differentiated amphibian cells, genetic totipotency was not demonstrated. Thus, the results from specialized somatic nuclei permit two interpretations: either irreversible genetic changes accompany somatic cell specialization and prevent totipotency, or the genetic potential of advanced cell types has not yet been adquately tested. Our reasons for favoring the latter possibility were based on the following three observations: (1) adult germ cell nuclei transplanted into enucleated eggs supported at best the development of a feeding tadpole (DiBerardino and Hoffner, 1971), yet such nuclei are destined to form mature sperm that will participate in complete normal development; (2) most nuclear transplants derived from advanced cell types exhibited chromosome aberrations that are associated with their abnormal development and arrest (DiBerardino, 1979); and (3) these chromosome abnormalities follow the observed asynchrony that exists between the transplanted nucleus and the egg nucleus in the egg cytoplasm with respect to the chromatin decondensation–condensation cycle, DNA synthesis and mitosis (Graham *et al.*, 1966; DiBerardino and Hoffner, 1970; Leonard *et al.*, 1982). Thus, this asynchrony reflects an inability of the chromosomes from advanced cell types to alter their behavior to that normal for the rapid cell cycle of the egg.

The standard host for nuclear transplantation has been the egg, and of course this is the host that was originally proposed by Spemann (1938). However, we hypothesized that the expression of the genetic potential of specialized cells might be enhanced if the donor nuclei were first conditioned in the cytoplasm of oocytes (DiBerardino, 1980; Hoffner and DiBerardino, 1980). The rationale for this proposal was based on the fact that the oocyte cytoplasm normally prepares its own chromosomes to participate in fertilization. Perhaps the same molecular components might prepare the chromosomes of injected nuclei to participate more normally in the activated eggs and during embryonic development. This possibility was supported by observations that nuclei transplanted into oocytes behave differently from those injected into eggs (see section on chromosome studies). Nuclei from advanced cell types injected into oocytes would not be forced into a period of rapid DNA synthesis and mitotic cycling with which they were not able to synchronize.

First, we determined whether somatic nuclei of embryos would, in fact, function during embryogenesis after residing in oocyte cytoplasm. To obtain oocytes at first meiotic metaphase female frogs were primed with an intraperitoneal injection of one half of a pituitary gland and 8 hr later the frogs were injected with the appropriate dose of pituitary glands for the season plus 1.5 mg of progesterone. The addition of the priming dose to the normal dose causes the oocytes to reach the uterus prematurely, *i.e.*, approximately in 24 hr (Elinson, 1977). These oocytes encased in jelly display in the animal pole the first black dot, indicative of the first meiotic metaphase. At this time, the oocytes are not activatable, but become so about 24 hr later as they mature *in vitro* (18°C).

A single blastula nucleus or an endodermal nucleus from a tail-bud stage embryo was injected into the animal hemisphere near the equator region of oocytes at the stage of first meiotic metaphase. Approximately 24 hr later, the matured oocytes were activated by penetration of a glass microneedle. Within 10 min the second black dot, indicative of the second meiotic metaphase of the oocyte, appeared and was removed microsurgically with a glass microneedle. Cytological studies revealed that transplanted embryonic nuclei formed metaphase chromosomes on newly induced spindles in concert with the behavior of the oocyte nucleus during first meiotic metaphase (Fig. 1A, B). When the matured oocytes were activated, the transplanted somatic nucleus transformed into a pronucleus. In a parallel series of nuclear transplants we found that the transplanted somatic nuclei could support development through embryogenesis (Fig. 1C; Hoffner and DiBerardino, 1980). Thus, somatic

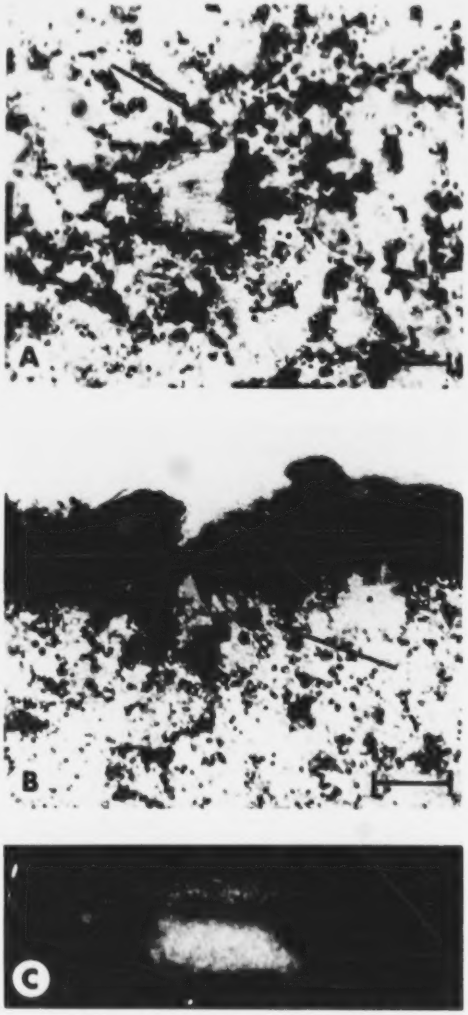

FIG. 1. **Oocyte that was injected with a single somatic nucleus at first meiotic metaphase. (A and B) Oocyte fixed 8 hr after nuclear transplantation. Scale bar, 10 μm.** (A) injected nucleus has transformed into metaphase chromosomes aligned on a spindle located approximately one-third down from the animal pole. (B) Oocyte metaphase chromosomes are aligned on meiotic spindle located just beneath surface coat of animal hemisphere. Note difference in size of spindles in A and B. The spindles of injected nuclei are 1.3 to 1.6 times longer (pole to pole) than the hosts' spindles. (C) Nuclear transplant derived from oocyte host (first meiotic metaphase) injected with a tail-bud endodermal nucleus. Length approximately 6 mm. (From Hoffner and DiBerardino, 1980.)

nuclei can reversibly respond to cytoplasms directing either meiotic or mitotic events. This result indicated that nuclei of differentiated cells could now be tested in oocytes to determine whether conditioning in the oocyte cytoplasm would result in enhanced expression of genetic potential.

Erythrocyte nuclei

For our test of the developmental potential of a differentiated cell nucleus, we chose the erythrocyte for two reasons: (1) identification of an erythrocyte is unequivocal under the stereomicroscope (100 × mag) because of its oval shape and the presence of hemoglobin; and (2) the erythrocyte is a noncycling and terminally differentiated cell, and one that is almost transcriptionally quiescent. Therefore, any development obtained from such transplantation tests would be certain to result from a nucleus of a differentiated cell. It should be noted that the erythrocyte is the least active in RNA transcription of the differentiated cell types listed in Table 2. For example, although only 0.06% of skin cells in culture incorporate low levels of ^{3}[H]thymidine, precursors of RNA and protein synthesis are incorporated efficiently (Reeves and Laskey, 1975).

One series of experiments used oocyte hosts (Fig. 2) and the other series, egg hosts, each injected with adult erythrocyte nuclei. Some of the blastulae derived from these nuclear transplants were used to provide donor nuclei for a second transplant generation. These blastula nuclei were injected into enucleated eggs and nuclear clones were produced (data presented in DiBerardino and Hoffner, 1983). None of the transplants from the egg series developed beyond the earliest gastrula stage. However, among the 12 clones produced from the original oocyte series, 8 clones (67%) contained members that proceeded to postneurula stages, and 6 clones (50%) had members that attained swimming tadpole stages. As judged from the original population of 130 nuclei tested, at least 11 (8.5%) adult erythrocyte nuclei programmed for postneurula development, and at least 6 (4.6%) directed the formation of swimming tadpoles (Table 2). However, only 57% of the blastulae were cloned in retransplantation experiments, because the rest could not be accommodated within the time span of the experiment. Among the 9 remaining blastulae, 5 attained neurula and postneurula stages. It is probable that these transplants had some nuclei that would have expressed greater potential after retransplantation into second generation hosts (see section on chromosome studies).

Of the 18 larvae that formed in the clones, 10 attained stages 22–25 (stage seriation of Shumway, 1940). These swam vigorously, the hearts beat regularly, and blood circulated through the capillaries of surface tissue of the body and tail. They had well formed heads, eyes, nares, ears, mouth and suckers. Internally, the brains and spinal cords had differentiated into gray and white cellular components. The eyes had formed lenses and displayed neural and pigmented retinas. The guts had fashioned esophagi, stomachs and livers, and had undergone some degree of intestinal coiling and formed hindguts. Cartilage was present in the mouth region. The oldest tadpoles displayed larval pigment and the opercular fold was nearly or completely closed. Verification that these transplants derived from the erythrocyte nuclei was provided by recovery of the egg nucleus in the exovates and determination of chromosome and nucleolar number. These studies demonstrated that (1) adult erythrocyte nuclei have retained the genes to specify postneurula and tadpole development, and (2) conditioning these nuclei in the cytoplasm of oocytes leads to the most widespread activation of the erythrocyte genome ever obtained in an experimental system. In fact, the specification of the various cell types in these tadpoles requires

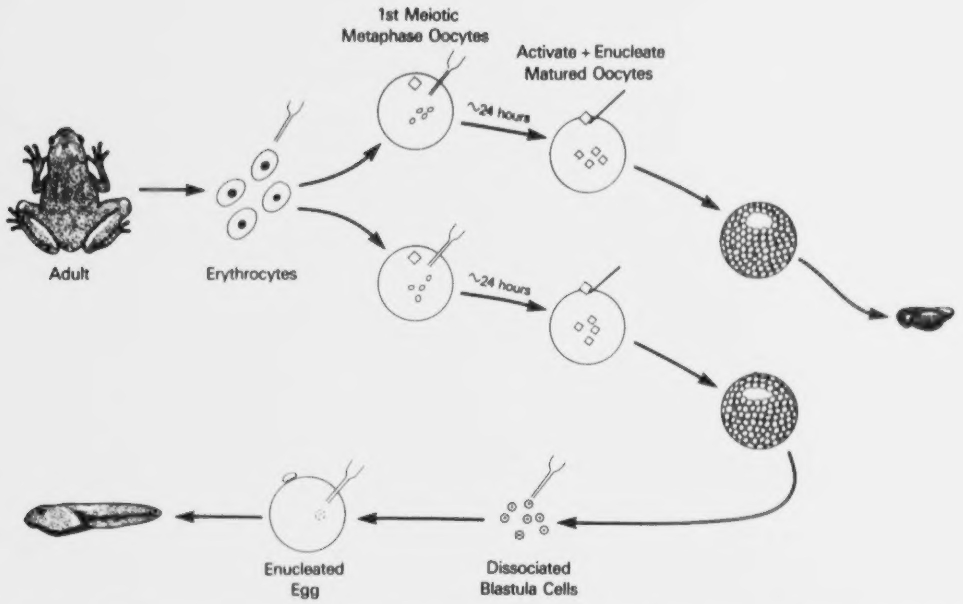
1st Meiotic Metaphase Oocytes
Activate + Enucleate Matured Oocytes
~24 hours
~24 hours
Adult
Erythrocytes
Enucleated Egg
Dissociated Blastula Cells

FIG. 2. Erythrocytes obtained by intracardiac puncture of adult *Rana pipiens* frogs were broken in distilled water by osmotic shock and microinjected into oocytes near the equator at first meiotic metaphase. Approximately 24 hr later (at 18°C) when the oocyte matured, the matured oocyte (egg) was activated by pricking with a glass needle, and the egg nucleus was removed microsurgically; (upper) original transplant generation, prehatching tadpoles resulted. In some cases, nuclear transplant blastulae were dissected and their animal hemisphere nuclei were transplanted singly into activated-enucleated eggs; (lower) first retransfer generation, swimming tadpoles resulted. Artwork by Betti Goren. (From DiBerardino *et al.*, 1984.) Copyright 1984 by the AAAS.

the activation of genes that never functioned in the cell lineage of an erythrocyte.

Centuplicate genomic replications

During normal development in *Rana pipiens*, there are at least 13 DNA replications and cell doublings between the first mitosis of the egg and the blastula stage and at least 19 cell doublings from the first mitosis to the postneurula stage (Sze, 1953). Thus, the genome of erythrocyte nuclei that programmed for swimming tadpoles in the second transplant generation described above underwent at least 32 DNA replications and cell doublings following the initial nuclear transfer into the oocyte cytoplasm. These numerous replications contrast dramatically with their total absence characteristic of erythrocyte nuclei in the frog. We next performed serial transplantation of erythrocyte nuclei into oocytes and eggs for 6 or 8 transplant generations (Fig. 3) to determine whether or not the erythrocyte genome was limited in its replicative ability (Orr *et al.*, 1986). Seven nuclear transplant blastulae of the first transplant generations were selected as donors to initiate the serial nuclear transplantation experiments, 5 derived from erythrocyte nuclei of 3 diploid adults and 2 from erythrocyte nuclei of 1 triploid juvenile frog, resulting in 7 nuclear lines. Even in the 6th and 8th transplant generations, the mitotic descendants of the erythrocyte nuclei were capable of supporting blastula development in 47–90% of the injected hosts. At this time the experiments were terminated and there was no evidence of a decrease in the capacity of the erythrocyte genome to continue

DNA replication and to participate in the cell cycle. Among the 7 nuclear lines, 3 lines contained members that developed up to neurula stages, one line's members proceeded to postneurula stages, while 3 other lines contained members that attained tadpole stages. Thus, the blastulae in the 8th transplant generation are derived from erythrocyte nuclei that have replicated their genomes and traversed mitosis at least 104 times, while the erythrocyte genomes that supported postneurula and tadpole development replicated their genomes at least 110 times. We conclude that once the genome of noncycling and terminally differentiated erythrocytes is activated by its progression through the cytoplasm of the oocyte and activated egg, it maintains its potential not only for replication but also for directing the complex functions of embryogenesis in excess of a hundred (centuplicate) cell cycles. In addition, the formation of postneurulae and tadpoles demonstrates that a large portion of the erythrocyte genome was retained.

The nucleated erythrocyte has served as a model to study gene activation and reversal of gene function, because it is the least transcriptionally active cell type known. Only one other experimental system, namely cell fusion, has led to significant genomic activation in erythrocytes. Chick erythrocytes fused with cultured mammalian cells synthesized chick DNA as well as certain chick RNAs and proteins (reviewed by Harris, 1974; Ringertz and Savage, 1976). However, the cell fusion system differs from the nuclear transplant system in at least two ways. First, the spectrum of gene products synthesized in the cell fusion experiments is limited compared to those required for the development of tadpoles. Second, unlike the chromosomes in the nuclear transplants, the chick chromosomes suffered pulverization during the first cell cycle (Harris, 1974; Ringertz and Savage, 1976) or at best were progressively lost after the first mitotic cycle (Kao, 1973). Thus, the euploid tadpoles derived from frog erythrocyte nuclei demonstrate not only the most widespread activation of the erythrocyte genome, but also the longest survival of the genome (DiBerardino and Hoffner, 1983; DiBerardino *et al.*, 1986; Orr *et al.*, 1986).

The ability of the frog erythrocyte genome to survive at least 110 replications and cell cycles is relevant to the problem of cells aging in culture. It has been consistently shown that normal cells in culture have a finite life span, and this applies even to embryonic cells (reviewed by Phillips and Cristofalo, 1983). This phenomenon has been interpreted to reflect the aging process. Thus, it is significant that the genome of the terminally differentiated erythrocyte can sustain centuplicate replications. Moreover, the erythrocyte genome retained all the genes required for tadpole development over the course of 110 cell generations, whereas the cell culture system is limited in the spectrum of gene products and cellular phenotypes. We, therefore, suggest that serial nuclear transplantation in amphibians is a good model system for studying whether eukaryotic genomes age in an *in vivo* embryonic environment that is primed to support DNA synthesis, mitoses and cellular differentiations.

Feeding tadpoles

As discussed above, the tadpoles derived from differentiated nuclei (Table 2) demonstrate the important conclusion that at least some of the test nuclei are genetically multipotent, *i.e.*, they contain the subset of genes required for early tadpole development. Nevertheless, the tadpoles in these studies failed to feed and express the traits required for progression beyond the early tadpole stages.

Recently, triploid erythrocyte nuclei from juvenile frogs of *Rana pipiens* were shown to direct the formation of feeding tadpoles that survived up to a month and

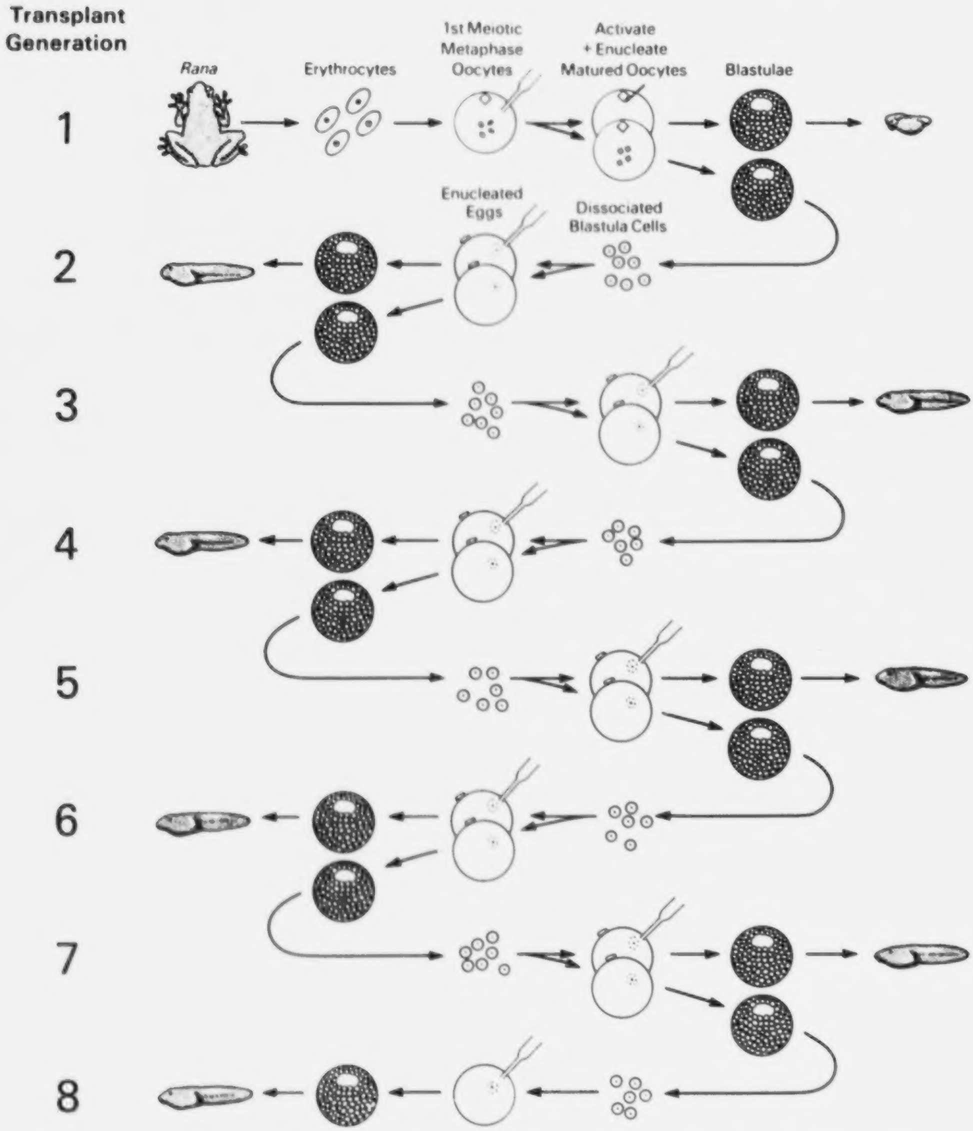
Transplant Generation
Rana
Erythrocytes
1st Meiotic Metaphase Oocytes
Activate + Enucleate Matured Oocytes
Blastulae
Enucleated Eggs
Dissociated Blastula Cells
1
2
3
4
5
6
7
8

FIG. 3. Serial transplantation of erythrocyte nuclei through eight transplant generations. Artwork by Betti Goren. (From Orr *et al.*, 1986.)

had differentiated hind limb buds (Table 2; DiBerardino *et al.*, 1986). In the first transplant generation, 31% of the injected oocytes (51) developed into blastulae (data presented in DiBerardino *et al.*, 1986). Four of these blastulae provided donor nuclei for enucleated eggs of the second transplant generation, and 4 nuclear clones were produced. Three of the 4 clones produced tadpoles that fed and 19 (21–67%) developed into tadpole limb stage I. They displayed the characteristics of stage I (stage

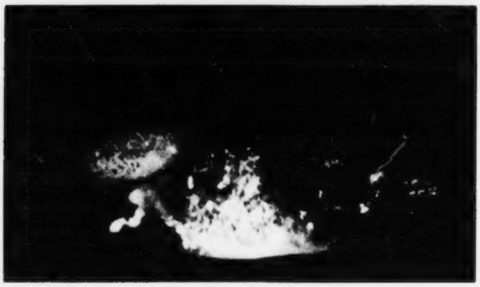

FIG. 4. **Triploid nuclear transplant tadpole from a triploid blastula nucleus derived from an erythrocyte of a triploid juvenile frog.** This tadpole was Taylor-Kollros stage III, 11-mm body length, and 1 month old. Arrow denotes one of a pair of hind limb buds. Below the hind limb bud is the rectum containing feces. Material adherent to the mouth is a remnant of food. Note patches of melanophores have covered the gill region and spread ventrad to the heart region. White spots are yellow chromatophores. (From DiBerardino *et al.*, 1986.)

seriation of Taylor and Kollros, 1946), namely, disappearance of the oral sucker elevations, formation of the rows of labial teeth, increase in chromatophores that extended progressively ventrad and the formation of a pair of hind limb buds. Nine tadpoles from 2 clones progressed into stage II. In this stage the hind limb bud increased in length, and the lateral line system on the dorsal surface of the head became evident. Also, the melanophore patches covering the gill regions spread into a narrow band ventral to the heart. Finally, 2 members in 1 clone progressed into tadpole stage III, the stage at which the length of the hind limb bud equals its diameter (Fig. 4). Of the tadpoles that developed into limb bud stages, 9 survived 2–3 weeks and 10 survived 3–4 weeks starting from the beginning of development. The length of survival of the feeding tadpoles is three times that of *Rana* nuclear transplants derived from diploid adult erythrocyte nuclei (DiBerardino and Hoffner, 1983) and greater than three times that of *Xenopus* nuclear transplants listed in Table 2. Chromosome counts made on the donor frog tissues, donor blastula nuclear transplants and all the tadpoles revealed the cells to be triploid (39 chromosomes), thus documenting that the feeding tadpoles were derived from the triploid erythrocyte nuclei. These results show that the erythrocyte genome of juvenile frogs can suport the development of an independent organism capable of feeding and growth. This is a critical stage, because continued development and further differentiation of the tadpoles is dependent on an external source of nourishment.

An important factor that must be considered in evaluating the nuclear transplants is the possible contribution of maternal RNAs and proteins to their development. It is known that the contribution made by the transplanted nucleus is significant, because eggs lacking a functional nucleus do not develop beyond the blastula stage (Briggs *et al.*, 1951). Nevertheless, there is not enough information at this time to evaluate exactly the role of maternal RNAs and proteins in the development of nuclear transplants. Thus, the survival of the nuclear transplants as an independent organism is quite important, because maternal products are presumably utilized and degraded within a restricted early period.

WHAT LIMITS TOTIPOTENCY?

Microscopic examination of nuclear transplant tadpoles derived from erythrocyte nuclei revealed that they possessed the cell types and organ systems present in tadpoles derived from fertilized eggs. If diverse cell types can be directed by the genome of erythrocytes and the other differentiated cell types—and this is a major accomplishment—why then do the nuclear transplant tadpoles not attain adulthood? In tadpoles derived from erythrocyte nuclei morphogenesis of the organ systems is abnormal. The most obvious defects are the irregular shape and small size of organs; they are composed of cells that are larger than normal as though cell division were less frequent. Observations indicate that the irregularities of organ morphogenesis may be a consequence of delayed gastrulation. The most advanced tadpoles displayed normal gastrulation, but in all cases, they were slightly delayed in their rate of progression through gastrulation. During neurula and postneurula stages, they had slightly smaller structures than controls and continued to exhibit a delayed rate of differentiation. For example, they initiated feeding about 1–2 days after the controls. It is possible that retardation during gastrulation could lead to incomplete induction and consequent limitations in development.

The most important question to consider is what causes the apparent limitation in the totipotency of erythrocyte nuclei. There are two kinds of explanations—either irreversible genetic changes have occurred that prevent feeding tadpoles from maturing into fertile frogs or the nuclear transplantation procedure is still not adequate to reveal the complete genetic repertoire of specialized cell nuclei. Although information is not available at this time to choose between these alternatives, we favor the idea that we have not yet adequately revealed the genetic potential of differentiated nuclei.

The inability thus far to obtain fertile frogs from specialized cell nuclei via nuclear transplantation into oocytes and eggs is not evidence for irreversible genetic changes. In principle, only losses in the DNA sequences of the genome would be irreversible. Such cases, so far as is known, occur as a regular event in only a few species, *e.g.*, in some protozoans, nematodes and insects (Klobutcher *et al.*, 1984), as well as in the genetic rearrangement of certain lymphocyte genes (*e.g.*, Hood *et al.*, 1985). However, it has not yet been shown that nucleotide losses that limit genomic totipotency are either a general phenomenon or occur in the nuclei that have been used as donors for nuclear transplantation experiments.

The most likely explanation for failure to obtain fertile frogs from specialized nuclei is that the nuclear transplantation procedure as now performed is still not adequate. The fact that several modifications in the procedure have led to enhanced expression of the genetic potential of somatic nuclei supports this view. First, the production of nuclear clones from blastulae of the first transplant generation has resulted in a larger percentage of successful transplants and more advanced development (Gurdon, 1962*b*; King and DiBerardino, 1965) due mainly to the sorting out of the fittest nuclei, those with the favorable karyotypes (DiBerardino and King, 1965; DiBerardino and Hoffner, 1970; DiBerardino, 1979). Second, inclusion of spermine in the nuclear transplantation medium, together with a reduction in the temperature (11°C) at which the operation is performed, led to a significant increase in the percentage of normal tadpoles derived from late gastrula and tailbud endodermal nuclei (Hennen, 1970). Third, exposure of embryonic neural ectoderm or notochord nuclei to protamine or polyarginine during transplantation resulted in fertile adults (Brothers, 1985). Fourth, residence of erythrocyte nuclei in the cytoplasm of maturing oocytes led to enhanced expression of their genetic potential (DiBerardino and Hoffner, 1983). Finally, it should be noted again that the theoretically totipotent adult germ cell nuclei have not resulted in development as advanced as that obtained with erythrocyte nuclei (DiBerardino and Hoffner, 1971). We have not yet determined whether the use of oocytes as hosts for the germ cell nuclei would obviate this difference, but it is important to test this point. Other factors, specific for germ cell nuclei, may also account for this difference.

Recent investigations have indicated that in the mouse normal development requires the presence of both the female and male pronuclei. Embryos with 2 female or 2 male pronuclei cease development early in embryogenesis (McGrath and Solter, 1984*b*; Surani *et al.*, 1984). These investigators have concluded that the female and male pronuclei are not functionally equivalent and suggest that a differential imprinting of the genome occurs during gametogenesis. This difference is reversible, however, because homozygous uniparental mouse embryos can develop in chimeric combinations with normal embryos and even develop into fertile adults that produce normal gametes derived from the uniparental genome (Anderegg and Markert, 1986). These authors suggested that the cases of totipotency stem from reimprinting the genome during gametogenesis. In amphibians gynogenetic diploids produced by supression of the second polar body can develop into fertile adults. These cases include *Rana pipiens* (Richards and Nace, 1978) and *Xenopus laevis* (Reinschmidt *et al.*, 1985). Thus, either differential imprinting of amphibian parental genomes does not occur, or, if it does, it is easily reversed.

IS GENETIC TOTIPOTENCY OF DIFFERENTIATED CELLS A TENABLE HYPOTHESIS?

An irreversible genetic mechanism for cell specialization was proposed a century ago (Weismann, 1892). The support for and against this hypothesis has cycled, with opinion often influenced by the organism studied and the techniques available. Recall that Spemann's view in 1938 was that every cell may be totipotent. Let us conclude this review by considering whether genetic totipotency of differentiated cells still remains a tenable hypothesis. Most of the evidence available today indicates that a large portion of the genome is retained in specialized cells. For example, the activation of dormant genes in specialized cells is a relatively common phenomenon, consistently demonstrated in several experimental systems including cell cultures, transdifferentiation, heterokaryons and cell hybrids, and cancer (reviewed by DiBerardino *et al.*, 1984). In these cases, the activation of dormant genes is accompanied by changes in the cell phenotype of specialized cells. Therefore, evidence indicates that silent genes can be maintained in the genome in the absence of expression, and that under favorable conditions they can be stimulated to function and to remain functional. These studies and others together with the results from amphibian nuclear transplantation argue strongly in favor of the hypothesis of genetic totipotency of at least some specialized cells.

The unique value of nuclear transplantation for this question is that it has the potential to test the entire genome and evaluate this problem in the context of a functioning organism. An alternate approach for studying the question of nuclear equivalence is available, namely DNA sequencing, but this would be a herculean task and would still leave unanswered the functional significance of all the nucleotides in the organism.

My view at the present time is that the proper conditions for reversing the function of the entire genome of at least some differentiated cells can best be found in the nuclear transplantation procedure. The chromatin of differentiated cells consists of its own set of chromosomal proteins required for its specific phenotype. When its nucleus is transplanted into a new cytoplasm, its chromatin must undergo remodeling of the chromosomal proteins in order for the genes to function properly. There is evidence for the remodeling of chromosomal proteins in germ cell nuclei during spermatogenesis (Dixon, 1972), oogenesis (Masui *et al.*, 1979), and after fertilization (Poccia *et al.*, 1981). With respect to the enhanced expression of genetic potential displayed by erythrocyte nuclei transplanted to oocytes, we have speculated that some degree of remodeling of chromosomal proteins may have occurred (Leonard *et al.*, 1982; DiBerardino and Hoffner, 1983). Autoradiographic studies of Leonard *et al.* (1982) revealed that only 24% of adult erythrocyte nuclei transplanted to eggs synthesized DNA, and in these cases, only a small portion of the genome engaged in DNA synthesis. If, however, adult erythrocyte nuclei were first injected into oocytes that later matured and were activated, over 75% synthesized DNA, and over one half of these nuclei synthesized DNA in amounts similar to the egg nucleus. Thus, we proposed that exposure of the erythrocyte chromatin to oocyte cytoplasm leads to a change, that renders the chromatin capable of responding to the signals in activated egg cytoplasm that induce the synthesis of DNA. Earlier autoradiographic studies examined the nucleocytoplasmic exchange of nonhistone proteins when endodermal nuclei were transplanted to eggs. These studies revealed that during the first cell cycle there is an accumulation of cytoplasmic egg nonhistone proteins by transplanted nuclei and also a major loss of nonhistone proteins from the transplanted nuclei into the cytoplasm (DiBerardino and Hoffner, 1975; Hoffner and DiBerardino, 1977). Perhaps a similar but more effective nucleocytoplasmic exchange occurs in erythrocyte nuclei placed in the oocyte, not only because of a longer exposure to the cytoplasm, but also because the nuclei are exposed to additional factors not present in the egg. Perhaps a complete remodeling of the chromosomal proteins leading to fertile frogs would occur in an even earlier stage of oogenesis such as the diplotene.

My view is that the eukaryotes utilize various mechanisms for achieving somatic cell specialization. In a few cases, the mechanism can involve losses and rearrangements of DNA sequences (Klobutcher *et al.*, 1984; Hood *et al.*, 1985). If the nucleotide losses are shown to be essential DNA, then an irreversible genetic change has indeed ocurred that will restrict totipotency. However, most somatic cell types probably achieve their specialized state by mechanisms (*e.g.*, DNA methylation, chromatin structure and DNA-protein interactions) that control specific genetic expression without an irreversible change in the nuclear DNA (DiBerardino and Hoffner, 1970; DiBerardino *et al.*, 1984). Thus, this latter group may retain the same genomic repertoire as the zygote nucleus.

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Cell Adhesion as a Basis of Pattern in Embryonic Development[1]

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SYNOPSIS. The development of the modern methodologies of cell biology in the fifties and sixties and of molecular biology in the seventies and eighties has led to a reductionist view of embryonic development that centers on the cell and the gene as the functional units of development. The functional units in most inductive and morphogenetic processes in the embryo are not single cells, however, but rather are collectives of interacting cells that give rise to the tissues and organs. Can these methodological developments reconcile a molecular analysis with the fact that form arises epigenetically from the increasing number of embryonic cells during development? To answer this question one must link genetic regulation to mechanochemical processes that coordinate cell division, cell movement and cell death. Recent studies of cell adhesion suggest that one such link is provided by cell adhesion molecules (CAMs) that mediate cell-cell binding. These studies suggest that CAMs are involved in defining cell collectives and their borders as they interact during inductive events in morphogenesis. Although CAMs cannot be considered the "cause" of induction, they play key roles among the complex causal chains of inductive interactions involving hormones and growth factors, extracellular matrix components and cellular receptors. We provide here a brief summary of modern developments in the field centered about the function of CAMs in morphogenesis and using recent experimental results in the developing feather as a paradigmatic example.

INTRODUCTION

It is remarkable that cells can assemble during development into patterned collectives that are the basis of animal form and tissue pattern. One of the key events in such activities is that of adhesion between cells. The earliest experimental analyses of the ability of isolated cells to form normal tissue patterns by adhesion were performed on sponges. Wilson (1910) demonstrated that single cell suspensions from disaggregated mature sponges would not only reaggregate, but that they would then rearrange to form small functional sponge organisms. Later, Galtsoff (1925) demonstrated that the reaggregation was species-specific; cells from two different species of sponges would sort out and reform sponges typical of each of the species.

Holtfreter and his colleagues (Holtfreter, 1944; Townes and Holtfreter, 1955) demonstrated that cell suspensions prepared from a vertebrate embryo would also undergo a sorting out process to yield a variety of histologically identifiable tissue structures. In this case, a fully formed embryo was not reconstituted, but identifiable gut, muscle, nerve, and skin tissues reformed from single cell dispersions of amphibian embryos. Similar studies by Moscona and his co-workers (Moscona and Moscona, 1952; Moscona, 1974) on the aggregation of cell suspensions from embryonic chicken tissues subsequently demonstrated that this was a general phenomenon at later stages of development.

A number of attempts have been made to understand the basis of these phenomena. For example, Steinberg performed a series of experiments in which cell suspensions from a number of tissues were mixed, allowed to aggregate, and then sort out to form tissues (Steinberg, 1970). Not only did cells from different tissues sort out to reform recognizable structures, but there was a consistent pattern in the relative topography of the aggregates. When two cell types were intermixed, they would always sort out with one cell type in the middle of the aggregate and the other cell type on the outside. Moreover there was a

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hierarchy to this behavior: cell types could be arranged in a list such that a given cell type would be wrapped around any cell type that was lower on the list, and be enwrapped by any cell type that was higher on the list. These results were summarized in a differential adhesion model (Steinberg, 1978) that used the formalisms of equilibrium thermodynamics and surface chemistry of fluids without specifying the actual molecules and without requiring cell addresses. While adequate as a provisional explanation, this model did not explicitly account for the kinetic, non-equilibrium nature of cellular processes.

In contrast, Sperry developed a different type of model after analyzing the development and regeneration of retinotectal maps in amphibians. He discovered that the point-to-point specificity of the connections of the optic nerve to the tectum was regenerated after damage to the optic nerve and suggested a chemoaffinity hypothesis to explain these results (Sperry, 1963). According to this hypothesis, the retina and optic nerve each express unique chemical markers (down almost to the level of individual neurons) which would match chemical markers on the tectum. If this were true, one might expect that detailed mapping is due to sorting out of the chemical affinities of the matching pairs of markers.

With the exception of the work on differential adhesion, which was non-committal about the molecules involved, the experimental results described above were interpreted to mean that there exist a large number of tissue- or position-specific adhesion molecules. These molecules would be expressed in the same way that other tissue-specific cytodifferentiation products are expressed, as part of a terminal tissue-specific spectrum of gene expression and would by their specificity and position assure form and tissue pattern.

One way of testing this idea is to identify and characterize molecules carrying out cell adhesion (Edelman, 1983). A molecular analysis of such cell adhesion molecules (CAMs) not only would be expected to give insights into how tissues are formed, but also might possibly provide a means of defining mechanisms of pattern formation. In the last decade, CAMs have been identified as a result of a variety of assays (Brackenbury *et al.*, 1977; Hyafil *et al.*, 1980; Damsky *et al.*, 1983; Gallin *et al.*, 1983; Imhoff *et al.*, 1983; Yoshida-Noro *et al.*, 1984; Gumbiner and Simons, 1986). When the isolated CAMs of different specificities were analyzed in terms of expression and tissue distribution, they turned out not to be expressed just in organ or tissue-specific patterns (Crossin *et al.*, 1985). Instead, they are expressed throughout development in a large number of specific locations in the embryo (Thiery *et al.*, 1982; Edelman *et al.*, 1983; Thiery *et al.*, 1984; Chuong and Edelman, 1985*a*, *b*; Crossin *et al.*, 1985; Vestweber *et al.*, 1985; Damjanov *et al.*, 1986), marking formation of cell collectives (early and late) and of borders between such collectives (see Edelman, 1986, for a review). Thus, CAMs would seem to be serving a function other than assembly according to positional specificity at the individual cell level within a tissue or organ. A number of other experiments showed that if CAM binding was perturbed it resulted in altered morphology (Buskirk *et al.*, 1980; Gallin *et al.*, 1986); conversely, alterations of morphology were found to result in changes in CAM expression (Daniloff *et al.*, 1986). Indeed, a variety of genetic defects that lead to altered histology and morphology show alterations in CAMs (Edelman and Chuong, 1982; Rieger *et al.*, 1986). Thus CAMs are not only important in morphology as defined special gene products that control cell assembly, but they are also dynamically regulated.

A clear-cut role for the CAMs in the generation of the pattern of organs and tissues in the embryo is apparent when the pattern of expression of the CAMs is compared to the inductive pattern-forming events that have been defined by embryonic manipulations: among other functions, CAMs appear to link cell groups that are undergoing reciprocal inductive interactions (Edelman *et al.*, 1983; Chuong and Edelman, 1985*a*, *b*; Crossin *et al.*, 1985). This in turn suggests that one of the major epigenetic factors in development is the capacity of cells to form cooperative populations with definite shapes and borders; these linked collectives have properties different from dispersed collections of individual cells. This provides the basis for new ideas on pattern formation and its genetic control through CAMs.

THE NATURE OF THE CELL ADHESION MOLECULES

We will restrict discussion here to two cell adhesion molecules that are present in the developing animal throughout early embryogenesis and postnatal development. These are designated primary CAMs by virtue of this property and because of their widespread distribution (Edelman, 1984*c*). There are also secondary CAMs, the expression of which is much more limited, both temporally and spatially (Edelman, 1984*c*), particularly during tissue formation.

The extensively studied primary CAMs are L-CAM (liver cell adhesion molecule) and N-CAM (neural cell adhesion molecule), each named for the tissue from which it was originally isolated. The complete protein structures of both CAMs have been deduced from analysis of cDNA clones and they are completely unrelated to each other in protein structure (Cunningham *et al.*, 1987; Gallin *et al.*, 1987). Consistent with this finding, N-CAM and L-CAM do not bind to each other. Both CAMs are intrinsic membrane glycoproteins that are expressed on the plasma membrane. L-CAM is a single polypeptide species of Mr = 124,000, containing four asparagine-linked oligosaccharides and it mediates calcium-dependent adhesion between cells (Cunningham *et al.*, 1984). Mammalian homologues of L-CAM have also been isolated from a variety of mouse (Hyafil *et al.*, 1980), human (Damsky *et al.*, 1983), and dog (Imhoff *et al.*, 1983) tissues. L-CAM is found on virtually all epithelial cells of the developing and adult organism (Thiery *et al.*, 1984). Molecular cloning experiments have demonstrated that the mRNA encoding L-CAM is the same size in all tissues tested, and that there are no more than three genes for L-CAM (Gallin *et al.*, 1985). The other CAM that we shall consider here (N-CAM) is in fact specified by only one gene (D'Eustachio *et al.*, 1986).

One of the remarkable features of N-CAM is that it is homologous in part to antibodies (Hemperly *et al.*, 1986*a*). It is thus a member of the immunoglobulin superfamily, and indeed may even be more closely related to the evolutionary precursor of that family than any other member (Cunningham *et al.*, 1987). N-CAM binding, in contrast to that of L-CAM, is calcium-independent (Brackenbury *et al.*, 1981). N-CAM is expressed as at least three distinct polypeptides that arise by differential RNA splicing from a single gene (Hemperly *et al.*, 1986*b*; Murray *et al.*, 1986). This splicing event changes the relation of a particular N-CAM polypeptide to the cell membrane by altering the nature of the carboxyl terminal end of each in a different way (Fig. 1). It does not change the extracellular binding portions, which are identical. Thus the constant part of different N-CAMs is the binding part whereas the part that varies is that related to the cell surface. This suggests that such variation alters N-CAM binding in terms of the control of groups of molecules on the cell surface. This is consistent with the proposal (Edelman, 1976, 1983) that cell surface modulation is a major basis for changes in cell interaction in development.

Two of the N-CAM polypeptides are

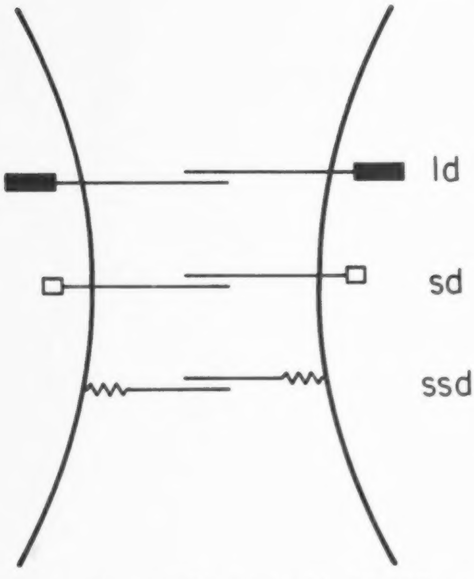

FIG. 1. Summary of the relationship of the different forms of N-CAM with the cell surface. Each of the three different polypeptides shares a common extracellular region that mediates cell-cell binding. The portions of the polypeptides that are associated with the cell membrane are generated by differential splicing of the mRNA that encodes the protein. One species has a large intracellular domain (ld) represented by the solid rectangle, one species has a small intracellular domain (sd) represented by the open square, and the third species has a small, surface associated domain (ssd) represented by the wavy line. These differences are consistent with differences in mobility and clustering of each type of chain at the cell surface.

specific to the nervous system; the third is widely expressed in the embryo, and it is possible that there are other N-CAM polypeptide species that have not yet been characterized. Like L-CAM, N-CAM is also asparagine-glycosylated. It has, however, a very unusual sugar (α2-8 polysialic acid) on certain of the asparagine-linked oligosaccharides (Rothbard *et al.*, 1982; Finne *et al.*, 1983). Although this sugar does not participate directly in N-CAM binding, it can influence it through the effect of its negative charge (Hoffman and Edelman, 1983). N-CAM on one cell binds to N-CAM on an apposed cell (homophilic binding, Fig. 1) and one would expect the amount on the cell surface to alter the thermodynamics of cell binding (Hoffman and Edelman, 1983). Thus, both the extent of sialylation and the concentration of N-CAM on the cell surface are factors that modulate the adhesion between cells expressing the protein.

We see that the two primary CAMs, which are expressed in many locations at many times, are not large repertoires of related molecules, but rather are discrete molecular species of different specificity that can yield a variety of binding states by cell surface modulation. How does this affect boundaries? To understand this, we must observe CAM expression during development.

EXPRESSION OF CAMs IN THE DEVELOPING EMBRYO

CAM expression is dynamic. L-CAM is expressed on the blastoderm cells of newly laid chicken eggs (Thiery *et al.*, 1984). N-CAM has also been detected at low levels in the chick blastoderm (Edelman *et al.*, 1983). As gastrulation proceeds to form the germ layers, the expression of the CAMs changes: cells leaving the blastoderm and ingressing through the primitive streak show much lower levels of the CAMs at their surfaces and become migratory mesenchyme. The remaining blastoderm cells in the epithelium continue expressing L-CAM. When the chick mesodermal cells form condensed structures (somites and notochord) they express N-CAM at higher levels again (Thiery *et al.*, 1982).

The notochord (and so-called chordamesoderm) is the primary inducer of the neural plate, that portion of the ectoderm that differentiates to form the nervous system. At this stage of development, we observe a motif that is repeated over and over again during development; two adjacent groups of cells (in this case notochord and ectoderm) each coupled by different CAMs (N-CAM and L-CAM) interact to change the fate of one or both cell collectives (Crossin *et al.*, 1985).

Inductive interactions between cell collectives were first defined in the classic work of Spemann and Mangold (1924) on the development of the neural structures of salamander embryos by transplantation experiments. This work defined the properties of the so-called organizer, a portion of the embryo formed in gastrulation which could induce whole embryos in the proper tissue environment. Since these studies, the concept of induction has been extended to local sites and organs, including, for example, the lungs, liver, pancreas, lens of the eye, and feathers. It is striking that immunohistological localization studies using antibodies to N-CAM and L-CAM have demonstrated that in each of these cases the two primary CAMs are expressed in characteristic fashion in the interacting cell collectives (Thiery *et al.*, 1982; Edelman *et al.*, 1983) and that the pattern of expression is consistent with cell linkage within each collective and border formation between two collectives (Crossin *et al.*, 1985).

To show how the CAMs may be related to inductive events and to demonstrate a connection between gene control, gene product, and the mechanochemistry of pattern, feather development is a particularly useful example. Indeed, one of the most striking examples of induction leading to formation of a new pattern is provided by the development of the feathers in the skin of the chicken. We shall choose this as our motif; ample evidence exists that if we can explain CAM relations in feather induction, the general features of the explanation will hold in other regions of induction. This is so because of the general role of CAMs and also because feathers show periodic patterns in their internal structure and in their distribution during both embryogenesis and histogenesis.

THE FEATHER: A PARADIGM OF PATTERN FORMATION

Let us begin by describing the development of the chicken skin. The vertebrate skin consists of the epidermis, which is of ectodermal origin, and the dermis, which is of mesodermal origin. Starting at about the sixth day of development, the skin of the embryonic chicken begins to form a definite pattern: a roughly *hexagonal* array of feather rudiments. These rudiments initially consist of a condensed mass of dermal cells covered by epidermal columnar epithelium (Wessells, 1985).

The development of the feather rudiments in the embryonic skin depends on inductive interactions between the dermis and epidermis. If the two tissues are separated prior to formation of the feather rudiments, neither one will develop its portion of the rudiment. More strikingly, if the epidermis and dermis are separated and then recombined with the appropriate tissue from another area of the skin, the feather pattern that is formed is characteristic of the dermis. The pattern of skin appendages depends on the dermis to the extent that dermis from the foot will induce the formation of foot scales in epidermis from the back or wing, which would normally form feathers (Rawles, 1963).

The epidermis is not simply a *tabula rasa* on which is imprinted a preformed dermal pattern; the pattern is under genetic control and is heritable. Extensive transplantation and recombination experiments with the *scaleless* mutant, which does not form foot scales or normal feathers, demonstrate that the patterns of feathers and scales that form from chicken skin are dependent on interactions between the epidermis and dermis (Sengel and Abbott, 1960; Goetinck and Abbott, 1963; Dhouailly and Sawyer, 1984). The two tissues together generate a pattern that neither tissue alone is capable of generating. Moreover, the capabilities and patterns differ in different species.

The distribution of CAMs in the tissues of the skin during feather development has been studied in detail (Chuong and Edelman, 1985*a*, *b*). A striking correlation between CAM expression and feather rudi-

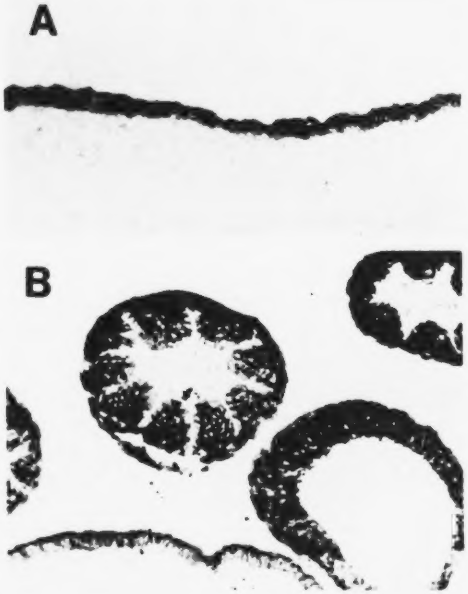

FIG. 2. Expression of L-CAM in the epidermis of the chicken skin. A. L-CAM is localized to the continuous epithelium of the epidermis of the skin from an 8-day chicken embryo. B. In the skin of a 13-day embryo, L-CAM is present on the epidermis (lower cell layer) and is also found on the epithelium of the feather rudiments, both before (right) and after (left) the barb ridges (see Fig. 3) have started to form. In both panels, transverse sections of the dorsal skin were stained with antibodies to L-CAM using an immunoperoxidase technique.

ment formation was found. This suggests that the formation and function of the tissues that give rise to the feather direct a particular regime of cell adhesion mediated by N-CAM and L-CAM. The epidermis expresses L-CAM throughout development (Fig. 2), and maintains a connected epithelial structure. The mesenchymal dermis cells of the 6-day embryonic skin that will induce the epidermis express neither CAM. As dermal condensations begin to form during induction, however, the cells that form the compact structure begin expressing N-CAM, thus linking the dermal cell collective via a cell adhesion mechanism different from that which links the epidermis. Each of the patterned expressions of the CAMs has functionally formed borders, between the epidermal cells that express L-CAM, the dermal condensation cells that express N-CAM, and the dermal mesenchyme cells that express neither CAM.

As the feather rudiment differentiates further (Fig. 3), the ectoderm folds into the dermis, forming a double layered epithelial collar, surrounding the dermal condensation that is differentiating to form a papilla. As a result of these mechanical changes accompanied by cell division and motion, the papilla and the collar interact inductively, causing the cells of the collar to proliferate. The resulting epithelial cylinder grows out of the skin to form the substance of the feather. Once again two interacting cell populations are coupled by the two distinct cell adhesion molecules, L-CAM on the epithelium and N-CAM on the papilla. Later still, as the barbs and barbules of the growing feather are differentiating, L-CAM and N-CAM are again expressed in a functional alternating pattern with L-CAM on collectives of epithelial cells that keratinize to form the feather substance and N-CAM on collectives of cells that eventually die and release the structure of the fully formed feather. The borders between N-CAM linked collectives and L-CAM linked collectives are converted by this final process into the edges of barbs and barbules.

This brief description indicates that, during the development of the feather, the two primary CAMs are expressed to form collectives and borders in at least three successive sets of interacting cell groups at different stages of differentiation. Indeed, it has turned out generally that, during embryogenesis, L-CAM and N-CAM are found in many of the interacting cell groups of inductive systems. The prevalence of this motif suggested that a primary regulatory factor in vertebrate development is the CAM-dependent coupling of cells into cooperative populations that will produce

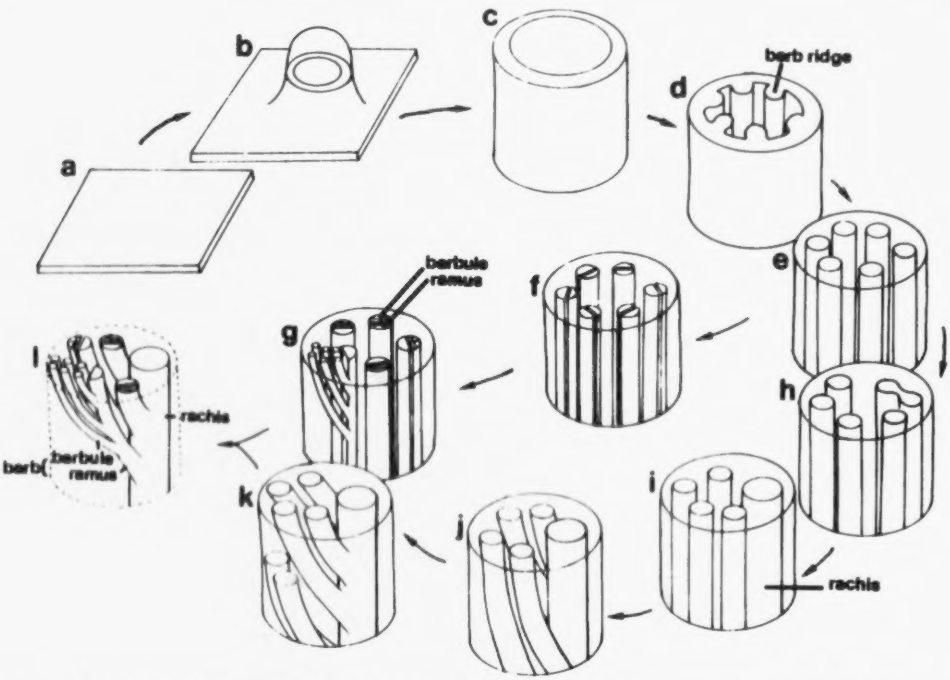

FIG. 3. Steps in the development of the highly ramified structure of the feather from a simple epidermal sheet. The epidermal sheet (a) evaginates to form a bud (b) and then a filament consisting of a cylinder of epidermis with a dermal core. The simple hollow cylinder (shown in section in c) develops ridges of epithelium (d) called barb ridges. These ridges are in turn subdivided (f, g, l) to form the barbs and barbules that are the substance of the mature feather. The barb ridges also fuse in some places with adjacent ridges (h, i, j, k) to form the branched pattern characteristic of the feather.

and respond to inductive signals. This provided an opportunity to test the so-called regulator hypothesis (Edelman, 1984*a*). According to this hypothesis, CAMs are special molecules under control of particular morphoregulatory genes that in turn regulate the structural genes specifying molecules (such as CAMs and the substrate adhesion molecules) that link cells to cells or cells and substratum. Such products are thus under different control than products of historegulatory genes (such as those that specify the keratins of the feather, for example). According to the regulator hypothesis, the emergence of CAMs as a result of local signals provided to their morphoregulatory genes might provide a basic mechanism necessary for further morphogenetic events that underlie the patterns of various tissues as well as animal form itself.

CAUSAL ROLE OF CAMS: PERTURBATION OF DEVELOPMENTAL PATTERNS

A major implication of this regulator hypothesis is that perturbation of cell-cell interactions in one tissue by antibodies to CAMs might not only alter the structure in that tissue, but might also disrupt induction-dependent changes in adjacent target tissues. If this were so, we might be able to advance beyond Spemann to the beginning of an understanding of the molecular regulation of pattern in induction. To test the regulator hypothesis, we therefore treated

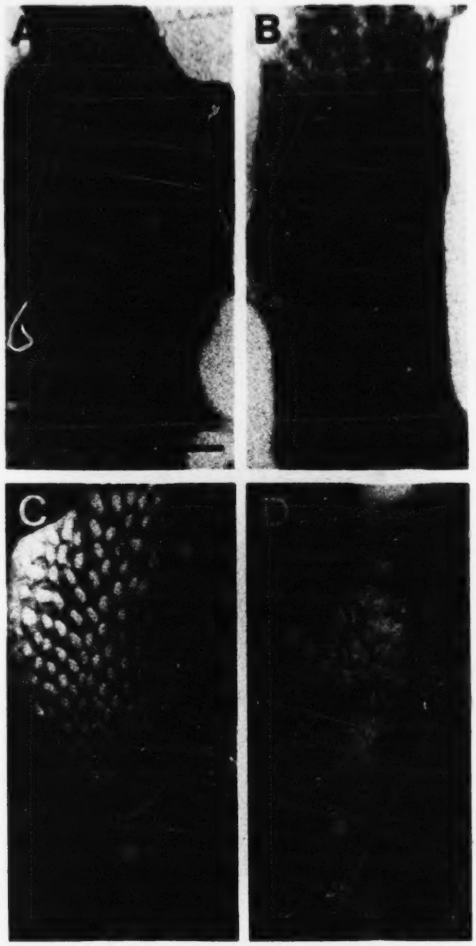

FIG. 4. **Whole mounts of 7-day embryonic chicken skin maintained in culture for 3 days (A, B) or 10 days (C, D). A and C were cultured in the presence of F_{ab}' fragments of antibodies from unimmunized rabbits; B and D were cultured in the presence of F_{ab}' fragments of rabbit antibodies against L-CAM. The three-day cultures were fixed in Bouin's fixative, stained with borax carmine and photographed by transillumination. The ten-day cultures were fixed and photographed with oblique incident illumination to visualize surface features. Scale bar = 1 mm.**

embryonic chicken skin in culture with antibodies to L-CAM (Gallin *et al.*, 1986). It is important to describe in a bit more detail the rationale of this experiment.

Just prior to the formation of feather rudiments, the skin consists of a layer of epidermis, which expresses L-CAM, and a layer of dense mesenchymal dermis. As the feather rudiment forms, the dermal cells follow one of two developmental pathways; they either become incorporated into dermal condensations, tightly packed cell groups that express N-CAM, or they remain as part of the loose mesenchyme and do not express N-CAM. The condensations are associated with epidermal placodes, the induced epithelial structures that take on a transient thickened morphology and show hexagonal packing in a developing feather field. The combination of a placode overlying a dermal condensation is called a feather rudiment (see Figs. 2, 3); this structure will develop and differentiate to form the feather and its associated generative structures.

The feather rudiments of the dorsal skin of the chicken form in a roughly hexagonal pattern, even in skin that has been removed from the embryo and maintained in organ culture (Fig. 4A). Because the characteristic pattern of the multiple feather rudiments arises as a result of inductive interactions between epidermis and dermis and because the inductive events that give rise to the rudiments can occur *in vitro*, we were able to devise a technique for perturbing the L-CAM mediated interactions between the cells of the epidermis with antibodies and thus determine the effect of this disruption on the development of the feather pattern. The idea was to disturb *only L-CAM interactions* and then search *in L-CAM linked structures* (the inducing mesoderm) for a change in pattern. Such a change, if found, could not be ascribed to trivial mechanical factors (*e.g.*, loosening of cell contacts in the dermis) because there is no L-CAM in the mesoderm.

In our early experiments, the effect of F_{ab}' fragments of antibodies to L-CAM was dose-dependent. High concentrations of antibody completely disrupted the epidermal layer, yielding a dermis stripped free of epidermis. These explants did not develop any discernible pattern, and resembled dermis that was mechanically stripped of epidermis. When the concentration of anti-L-CAM was lowered to a level that left an intact epidermal epithelium on the explant, we were excited to find that a novel pattern appeared in the explants. Instead of a hexagonal array of circular rudiments, a series of stripes were formed (Fig. 4B). These stripes were not straight, but meandered; there was, however, local order in the sense that the stripes were often parallel, and the spacing between the stripes and other regions of condensation were fairly uniform.

Histological analysis of the perturbed skin cultures revealed that the morphological aberrations *were due to changes mainly in the dermis, not the epidermis.* The stripes observed in the whole amount were due to formation of stripes of dermal condensations instead of the normal circular collectives. In addition, we found that the density of cells between the condensations of the perturbed explants was much higher than that in the unperturbed culture. Just as striking, analysis of the spatial pattern of cell division by labelling dividing cells with pulses of tritiated thymidine demonstrated that the pattern of mitosis in the *dermis* had also been altered by the anti-L-CAM antibody treatment that had affected the linkage of cells in the *epidermis.*

Consistent with a dynamic role for CAMs in epigenesis, we also found that when cultures that were treated with specific antibody for the first three days were allowed to develop in culture for a further seven days after removing the perturbing antibody, the structure of the skin appendages that developed was also distinctively altered. Whereas the unperturbed cultures developed filamentous structures (Fig. 4C) consisting of a core of mesenchyme covered by a layer of cuboidal epithelium, the perturbed cultures developed cobblestone-shaped, plaque-like structures (Fig. 4D) that consisted of dense dermal condensations overlain by differentiated multi-layered squamous epithelium.

The gross morphology and histology of these long-term perturbed cultures is reminiscent of the development of foot scales, whereas the normal cultures had developed appendages that resembled feather rudiments. Thus, not only did perturbation of the L-CAM mediated linkages in the epidermis cause a change in the early *pattern* of appendages that were formed, but this perturbation in turn had long-term consequences for the *histological development* of the appendages, even after the perturbant had been removed.

The consequences of altering the milieu in which the cells of the skin are developing, in this case by altering the direct cell-cell interaction mediated by CAMs, were short term in affecting the morphological pattern that is evoked by the normal inductive interactions between epidermis and dermis, and long term in that the morphologically altered explants continue to develop histological patterns different from their normal fates. Thus, somehow the signals between cells linked to form two kinds of collectives, the borders of which are defined by different CAMs, were interpreted differently when one of the CAMs (L-CAM) was weakened in its binding function within one of the collectives (that of the epidermis). The perturbation did not just lead to disorder but changed an inductive pathway and subsequently a histological developmental pathway by epigenetic means at the molecular level. In other words, the genetic capabilities of the tissues had not been altered but the results of gene expression were changed.

PROSPECTS

These experiments, which provide an opportunity to relate gene expression of morphoregulatory molecules to tissue pattern, were based on the idea that the unit of regulative development is not the single cell, but consists of groups of cells linked

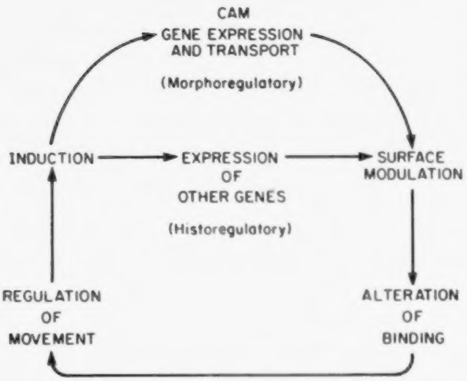

FIG. 5. Summary according to the regulator hypothesis (Edelman, 1984*a*) of the relationships of developmentally important cellular processes. Inductive interactions between tissues lead to changes in the expression of morphoregulatory genes controlling genes specifying CAMs and substrate adhesion molecules, and also to changes in historegulatory genes that control specific cytodifferentiation products (such as keratins in the feather). These effects are integrated through cellular modulation mechanisms to alter the mechanochemical properties of the cells, for example binding, mobility and mitotic response. The changes thus induced in the cellular milieux lead to another set of inductive interactions, and the cycle repeats with variations of expression of CAMs, of historegulatory gene products or both. In this way mechanochemical events leading to cell collectives are linked to developmentally important genes.

by CAMs and their appropriate associated extracellular milieux. A principle consequence of this assumption is that the molecules that constitute the local environment of the cell are crucial to regulative development because they are the basis for temporal and spatial cooperativity among collectively linked cells within a developing tissue. They provide some additional support for the regulator hypothesis, which may be encapsulated in a schema as shown in Figure 5. Although we have focused on the role of CAMs in inductive interactions, it is clear that the components of the extracellular matrix and the cellular receptors for those components, the molecules of the various morphologically distinctive junctional complexes, and growth factors, hormones, and receptors all may play vital roles in development (Edelman, 1985, 1986; Edelman and Thiery, 1985). It is also true that the roles of these various factors are intricately interwoven in a cooperative network of effects on the cells of the developing embryo that integrate the various primary cellular processes of development. The really significant new opening provided by the feather experiments is the demonstration that a surface protein under genetic control can dynamically influence pattern by altering the cellular mechanisms of pattern formation in a causal fashion.

A complete analysis of the role of the cellular milieux in development will entail description of a *network* of control, including the genetic control of expression of CAMs and extracellular matrix molecules, the cellular modulation of their expression and structure (Edelman, 1976, 1983), the consequences of the function of these molecules on cellular physiology, and the effects of these physiological consequences on expression of other genes (Edelman, 1984*a*, *b*). It is clear that a beginning has been made in discerning a pathway of control—gene to gene product to the cell collectives under the regulation of the gene product—thus providing a basic scheme (Fig. 5) for morphogenetically significant pattern. It is only by understanding such a scheme in detail that we can speak of a true science of molecular embryology. As other discoveries on the regulation of the signals released to express CAM genes and thus influence pattern are made, we may expect to extend the frontiers of molecular embryology and further understand epigenetic events in development and their evolutionary control.

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Inheritance of Pattern: Analysis from Phenotype to Gene[1]

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SYNOPSIS. The form and pattern of multicellular organisms are developmental phenotypes. They are long term processes rather than static structures. They involve myriad events at multiple locations. The efficient encoding of such phenotypes is analyzed here in two stages. First, the complex developmental behavior is broken down so it can be accounted for by cell or tissue rules. The most effective rules have the instantaneous character found in time-based differential equations. When integrated over time and space, the rules produce the behavior. Second, the cytological and nuclear basis of the rules is sought. One thus studies a complex phenotype in terms of its successive antecedent causes, refining understanding as one gets closer to the genome.

The approach is applied here to phyllotactic (leaf placement) patterns. Leaves may be alternating in a plane, whorled, or in a helical arrangement. In all three cases a new leaf forms as an arc-like bulge at a site apical to a small number of neighboring leaves. The leaf-forming sites are irregularities in the pattern of cellulose reinforcement in the surface of the apical dome. Two organ-level rules combine to produce new leaf sites. First, each established leaf develops a single reinforcement field, with gently curved reinforcement lines, on the region of the dome just above the leaf. Second, where parts of two or three such fields abut on the dome they combine to make the irregularity for the next leaf. Hence a given reinforcement pattern on the dome produces a leaf; the action of the leaves in turn reestablishes the reinforcement pattern. The cellular basis of generating a reinforcement field appears to be a cytoskeletal response to excessive stretch, brought on by rapid growth of adjacent leaf bases. The large scale patterns are thus traceable to cytoskeletal phenomena and from there to genes involving microtubular behavior.

INTRODUCTION

This essay consists of two parts. First, the general problem of analyzing the inheritance of pattern, in all organisms, is addressed. It will be concluded that analysis from the phenotype, via antecedent biophysical causes, toward the gene can be particularly effective. Second, this approach is applied to the study of the patterns found in plant shoots and flowers. It will be concluded that leaves are formed through cyclic biophysical activities in the surface plane at the tip of the shoot. These phenomena can be traced to cytoskeletal behavior and hence to the genome.

SPECIFIC DIFFICULTIES TO THE STUDY OF FORM AND PATTERN

The difficult aspects of studying the inheritance of form and pattern can be readily recognized. The outstanding one is that form and pattern are not simple tangible things. Rather, they are an end-product of a long progression starting at, or before, fertilization. The organism inherits the whole progression, not simply the end product. There are myriad pertinent events, occuring in a reproducible fashion. Furthermore, the events occur at specific locations over many microns, or millimeters, of space. Form and pattern will be called developmental phenotypes. They are continuous integrations, over much time and space. This distinguishes them from other kinds of phenotypes.

The distinction among phenotypes is seen in a simple two by two matrix. One axis determines whether the phenotype is small (described in terms of macromolecules) or big (described at the cell or organ level). The other axis deals with whether the phenotype is positive, concerning the presence of something, or is negative, concerning the lack of something. Developmental phenotypes are both big and positive. It is noteworthy that they are by far the least understood. The nature of understanding phenotypes in general will be briefly reviewed; then the best approach for developmental phenotypes will be addressed.

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Understanding means a "one to one" correspondence

Understanding a phenotype usually means finding a one-to-one correspondence between features of the genome, *i.e.*, base pair sequence, and features of the phenotype. This is relatively easy for the three non-developmental classes of inherited features. The presence or absence of an enzyme was the phenotype that enabled Beadle and Tatum to give rise to the field of biochemical genetics. They provided the famous duality, "One gene, one enzyme." The steps in between, now elaborated with removal of introns and with post translational modification, are not mysterious. The issue of one-to-one correspondence has gotten ever more exact. The interaction of proteins with other molecules is now analyzed in terms of point for point correspondence between details of surfaces of paired molecules, with precision at the atomic level (Smith *et al.*, 1986). This is the realm of reductionism where detailed molecular correspondence is safely anticipated. Small phenotypes, positive or negative, present no fundamental difficulties. The tactic "isolate and simplify" has been extraordinarily successful in clarifying the gene to phenotype relation at the molecular level.

The large, but negative, phenotypes are also relatively well understood, but within an obvious limit. The gene for winglessness in *Drosophila*, for example, could be studied in detail. The amino acid sequence of its wild-type product could be deduced. Its functional, or essential, parts could be ascertained. One could thus learn those specific features of the genome which, when absent, will cause failure of the wing to appear. The limit is that this does not tell us, in any satisfying way, what actually makes the wing arise in the wild type. The challenge of explaining a large and positive phenotype requires that two major things be accounted for: the sequence of the many contributing events, and their distribution in space.

Methods for one to one specification of position

Questions about position are readily handled within the macromolecular domain by specificity of molecular binding. Scaling up this concept to the point where cell-to-cell binding generates pattern is an important idea for development (Edelman, 1986, 1987). However, both animals and plants generate organs by the local folding of a coherent epithelium or epidermis, without major change of cell contacts, so there must be more to positional issues than specific binding by freely moving cells.

The best known proposal for specifying location on a large scale uses Positional Information, a concept originated by Wolpert (1971). This approach is "holistic" rather than reductionist. Molecular detail is foregone in return for an operational grip on the whole large scale situation. The idea is that position can be specified by a coordinate system, X and Y, as in analytical geometry. Rather than have each value on each axis be determined by a different gene product, which would presumably soon exhaust the coding capacity of the genome, the idea is that a continuous spatial gradient of single gene product, a morphogen, gives consecutive values along each axis. With two perpendicular morphogen gradients, a cell reads its position accurately in two dimensions. The model proposes that a transduction ensues whereby the cell does the appropriate thing for its position.

Because different things happen sequentially at the same location, a weakness of this approach is that the time sequence at a given location must also be specified. The ingenious economy evident in the handling of the position problem is not evident for the treatment of timing. Hence Positional Information, at least in this simple form, handles only one half of the problem. One must also deal efficiently with time sequences.

One to one specification of sequential activity

The way that sequential activity is inherited is no great problem, in principle, within the molecular domain. The complicated sequence of events that determines whether a virus will be latent or lytic is clarified in a recent book on Lambda (Ptashne, 1986). Sequence in time is readily explained at the molecular level by the progression of a polymerase in one direction from a promoter site. The subsequent transcription and translation of new polymerases or regulatory molecules then initiates transcription at new sites. Time is required to build up the proper concentration of a regulator to turn off, or on, certain initiation sites. The encoding of a specific time sequence of molecular activity is not difficult to envision.

It is tempting, therefore, to extrapolate from the valuable dictum of "one gene, one enzyme" to assume "one gene activation, one developmental event." The difficulty is that, in large scale development, there are far too many developmental events. There are more specific neuronal connections (10^{12}) made during the development of the human nervous system than there are base pairs (10^{10}) in the human genome (Johnson, 1987). There must be an economy in specifying the sequence of cellular events. There is no question that gene activity in the broad sense is ultimately behind all developmental phenomena. The questionable assumption is that sequential gene activation is a broad enough concept to account for the developmental sequences. There is long standing evidence that additional phenomenology is involved.

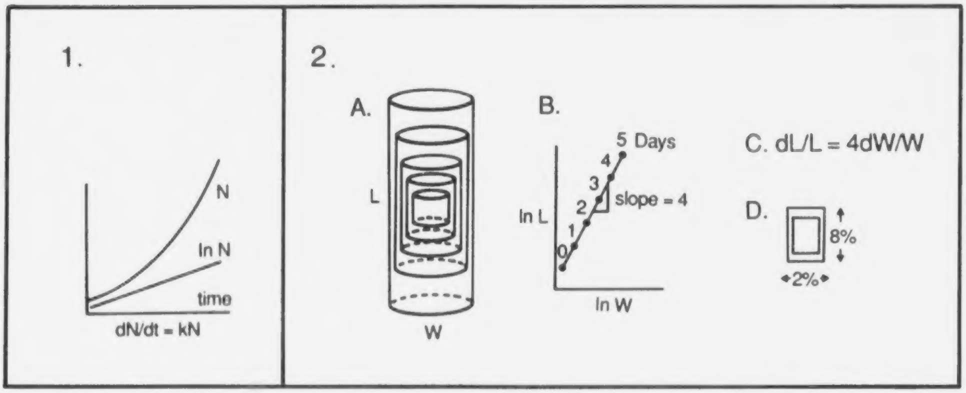
The unicellular marine alga *Acetabularia* can "decide" to produce a reproductive, rather than a vegetative, cap structure even in the absence of its nucleus (Puiseux-Dao, 1970). This shows that not all important developmental decisions are made at the level of concurrent gene activation. It is clearly possible to have sequential development while gene activation states are constant.

Gene states normally do change during development and it is useful to have information on stage-specific transcription or translation. The limitation here, however, is that a protein's appearance is important either to the concurrent development or to any later stage. By the same token, a given developmental process, such as the sequence of reactions of a sea urchin egg upon fertilization, is a function of many proteins, previously produced at various times. The sequence of their production need have no simple correspondence to the sequence of the developmental process in question. Thus the finding of molecular correlates does not necessarily provide a full explanation for sequential development.

In brief, there has been difficulty in accounting for spatial detail and progression of time sequence, as independent developmental problems. Various one-to-one concepts, found in positional information, sequential gene activation, etc., have not been entirely satisfactory. The major problem is that most one-to-one concepts simply relate one change to another and hence lack a built-in compounding property. This last is necessary if one is to encode, with high efficiency, a multitude of events. We turn now to another one-to-one concept, one which has this compounding property. Furthermore, it can deal with specification of time sequence and position concurrently.

A one to one relationship which has generative properties

This section advocates, for developmental studies, the use of another kind of one-to-one relationship, that between a time-based differential equation and its



FIGS. 1, 2. Specification of sequences by time-based differential relationships. FIG. 1. The exponential time course of increase in cell number N is the integral of the well known differential equation below. The constant k is the relative growth rate. FIG. 2. A progression in cell proportions, toward highly elongate form, is shown in A. Length is L and width is W. The slope on the double log plot in B is the coefficient linking fractional increment in length to fractional increment in width, as in equation C. This means that, for example, an 8% increase in length will always be accompanied by a 2% increase in width, as in D. (From Green and Poethig, 1982, with permission)

integral. This kind of coupling, when generalized in a biological context, can readily account for reproducible progressions in space and time. The coupling occurs in the form of cell rules where the cell makes a stereotyped response to a specific condition. This kind of coupling provides the necessary compounding feature to the casual chain linking genotype to phenotype.

One case of the efficient encoding of a sequence by a differential equation is well known. The exponential growth curve for a colony of cells is generated by integrating the equation $dN/dt = kN$, where N is the number of cells and k is a constant. A reproducible time course for population growth is tersely encoded (see Fig. 1).

This equation can be converted to a verbal form. It can be expressed as a rule which operates for a population over small (differential) time steps. The rule is that during each step the population size is increased by an increment proportional to the present population size. There is no difficulty in visualizing the biological reality and pertinence of the rule. On the one hand its integration leads to the reproducible population growth. On the other, the rule comes from the fact that the cells are asynchronous and have a common cell cycle duration. This duration, which determines the value of k and hence the exact form of the curve, reflects the speed with which the cells complete the cycle under the conditions at hand.

The verbalization of this rule allows recognition of the key features of useful, or generative, developmental rules. First, the rule couples an *action* (incrementing the population size) to a *condition* (the present size of the population). Both the condition and the response are in the same language (N). The rule looks neither forward nor backward; it is an isolated instantaneous coupling, pertaining only to "now." Also, the results of all previous activity, *i.e.*, previous increments, are carried forward as cell numbers accumulate. Thus an instantaneous action is based on current conditions, and all previous activity is carried forward. Thus one can interpret developmental progression as equivalent to mathematical integration, over time and space.

There are now two major "one to one's"

to be worked out: between the development and the rules and between the rules and the genome. It is evident that the rules need to be understood early in the analysis. To do so, it is easier to reduce the developmental progression to rules than to somehow start with the rules. The advantage is that reducing the phenomenology to rules is equivalent to differentiating an integral, *i.e.*, differential calculus. Starting with the rules, and proceeding toward the developmental sequence is doing integral calculus, a process with the complication of constants of integration and boundary conditions. Integral calculus is taught to us later, for a reason. Two more examples should make the reader at home with looking at development as an integration, and with studying it through differentiation.

The development of the giant internode cell of the pondweed *Nitella* involves the growth of a small tuna-fish-can shaped cell into a giant telephone-pole shaped cell (Green and Poethig, 1982). The final cell is several times broader than the original cell. The developmental phenotype here is thus a progression of ever more elongate cells, as shown in Figure 2A. The reduction of this sequence "to rule" is very easy. A double log plot of cell height vs. girth shows that the intermediate cell proportions fall on a straight line with a slope of about 4, as in Figure 2B. This means that growth in height and in girth can be treated as two compound interest rates, kept in a fixed ratio. The rate for height is four times that for girth as in Figure 2C, D. The simple relation works only if the rates are those for continuously compound interest.

The verbalization of this instantaneous activity is straight forward. Starting with a square, for simplicity, the rules say that the percentage increment in height will be four times the percentage increment in girth. Thus in one small step the height goes up, say, 8%, girth, 2%. (Actually the increments are infinitessimal, but in the same ratio.) Over the first time step this converts the square to a rectangle. In the next time step the rules are applied to the rectangle, not the original square, so the axial ratio of the cell quickly increases giving an ever more elongate outline.

The progression for cell shape has been reduced to a rule which dictates a constant bias in fractional extension, favoring height, for each interval. The remaining explanation of this phenotype must link this directionally biased growth to the genome. The nature of the connections comprising the link is illustrated in Figure 3.

The bias in stretch rate, height *vs.* girth, is explained by strong transverse reinforcement of the cell by cellulose (Fig. 3C). The reinforcement restricts the natural increase in girth. This connection is made through the physics of directionally reinforced cylinders. The transverse reinforcement is in turn a function of the transverse alignment of cytoplasmic microtubules whose orientation appears to govern that of the cellulose (see Gunning and Hardham, 1982). The maintenance of a transverse configuration of microtubules in the cortical cytoplasm of a longitudinally growing cell is a challenge yet to be explained. Strain alignment should pull the microtubules longitudinally. Possibly the microtubules maintain their alignment by adhering to the cell membrane and by minimizing their own girth through mutual sliding (Green and Poethig, 1982). At any event, there must be rules that keep the microtubules transverse. The composition of the microtubules, and their special behavior, is a function of proteins. At this point the causal chain reaches the domain where molecular phenomenology is relatively well understood.

From the diagram in Figure 3, there are 7 stages in understanding the final phenotype: shape progression of a single cell. The over-all chain is based on six one-to-one relations between successive stages. It is seen that the mode of conversion of one

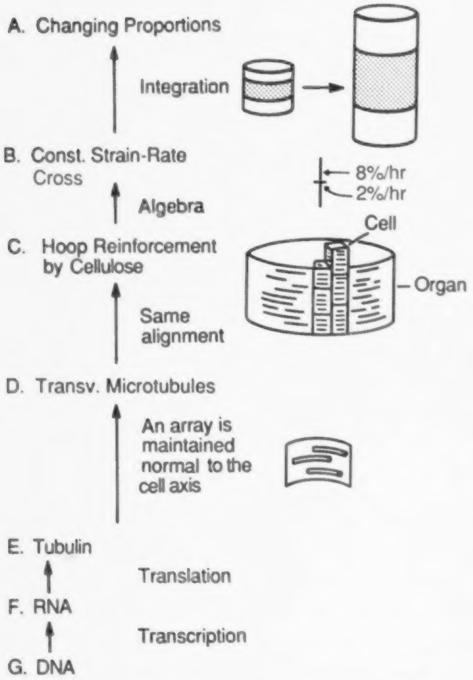


FIG. 3. Steps linking the simple developmental phenotype in Figure 2 to the genome. When read from top to bottom, the sequence is one of analysis. Read from bottom to top, the sequence is of apparent causation. A. The phenotype, cell elongation, is based on the repeated application of a directional strain-rate cross, a ratio of perpendicular growth rates, B. The cross is accounted for by the yielding properties of hoop-reinforced cylinders, as in C. The cellulosic reinforcement is apparently governed by transversely oriented microtubules, D. These stay transversely oriented presumably due to properties of tubulin and other proteins, E. These proteins arise from sequences in RNA and DNA, stages F and G. In this scheme of stages and conversions, protein properties stand less than half-way from the gene to the phenotype. The mechanism of conversion between stages changes markedly along the chain. (From Green and Poethig, 1982, with permission)

stage to the next varies along the chain. The one-to-one linear relationships, so basic between DNA and protein, give way to different kinds of conversions in the stages remote from the genome. Essential elements of some steps are physical. The last step, before the final phenotype, is the integration of a differential relationship.

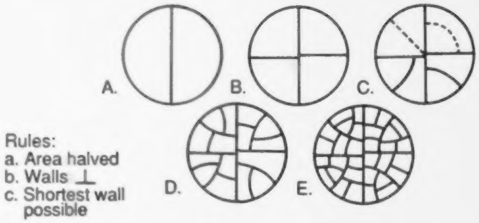


FIG. 4. Generation of a geometrically complex multicellular sequence by constant simple rules (after D'Arcy Thompson). This is the cleavage sequence in a flattened egg, explained by three rules. At each division a) cell area is halved, b) the new partition meets old walls at 90°, with only three walls meeting at a point, c) the new wall is the shortest that meets the other requirements. In C, the two dashed walls are not the shortest possible. The proper new walls are solid lines. Each quadrant always has one three-sided cell, an unexpected consequence of following the rules. (From Green and Poethig, 1982, with permission)

A long range one-to-one correspondence is thus fully understood only when all the intermediate conversions in the chain are known. When read from gene to phenotype, the chain shown in Figure 3 is causal. When read from phenotype to genome, the chain is seen in the advocated direction for analysis, particularly for the stages remote from the gene.

A final example illustrates the utility of "reduction to rule" for a developmental sequence at the tissue level. The example comes from Thompson (1942) and deals with the sequence of cleavage in a flattened egg. As shown in Figure 4, many partitions subdivide the original circular egg into a complex, yet reproducible pattern. The direct specification of the timing, location, and angle of each cleavage would of course require large amounts of information in the genome. Thompson's point is that the sequence, in both time and space, falls out from a short set of rules, the rules being constant throughout. The set of rules, or generative algorithm, is in the special instantaneous format where an action is coupled to a condition.

The rules are that a) the new partition halves the area of the parent cell, b) new partitions meet old walls at 90°, three at a point, and c) the new wall is the shortest one that meets the other two criteria. The rules apply to all cells, all the time. The time step is one cell cycle.

The first two cycles, A and B, are easy to anticipate. At the third, C, however, the solution is not obvious. The first inclination, to continue with a radial wall, violates rule one; the second inclination, to put in a circumferential wall, violates rule three. The oblique arc shown fits all requirements. Thereafter, the process continues to yield a configuration which ultimately has hints of a cortex/medulla arrangement. There will always be one three-sided cell in each quadrant. The constant rules breed increasing complexity because the boundary conditions, the cell outlines, keep changing. Thus one generates change from constancy, giving fundamental economy in encoding a progression.

There is no need to keep the rules in a simple fixed relationship. For example, in the present case, cells below a certain threshold size might always "change" rule #3 to call for the longest wall that would meet the other conditions. Such a shift in behavior could well require new gene products. The logical encoding of such behavior can nonetheless be reduced to constant rules. In the present example, the third rule would simply be rephrased to allow two conditions. It would read: if bigger than a certain size, make the shortest wall; if smaller than a certain size, make the longest wall. In this way the coupling of instantaneous relationships becomes flexible, as a flow scheme. Interactions between cells can also be incorporated. Whether new gene products are required for a given shift in behavior is a function of the response term. The shift from straight to curved walls at division three presumably would not require a shift in gene expression; a shift from shortest to longest wall presumably would. The format allows development to be broken down into its essential components without presupposing any details of mechanism.

DEVELOPMENT AS AN INTEGRATION: CONCLUSIONS

First, the genes pertinent to a given developmental process are all those responsible for the rules to be valid. This is bound to be a large number. Further, the sequence by which the pertinent genes are activated need bear no parallel to the sequence by which the development occurs. Nonetheless the tie in between the genome and development can be pursued effectively, by relating specific genetic changes to variations in the generative scheme.

A second conclusion is that the requirement to account for specificity in both space and time can be satisfied concurrently. The spatial features are specified by the sequential following of rules of a geometrical nature.

A third is that the generative rules, despite being strictly operational, constitute a sufficient explanation of what is going on. The rules generate the progression provided they can be followed. They will account for development just as Mendel's rules provided prediction for progeny ratios. Like Mendel's laws, the rules invite subsequent refinement for mechanism. Sufficiency, in the form of somewhat abstract rules, is gained while one is still lacking molecular specificity. The reciprocal trade-off is found in some molecular correlations in development. The molecule itself is known in detail. What it does, is often not. The molecule is "implicated" in the process; this connection does not, in itself, provide a sufficient explanation.

A fourth conclusion, related to the above, is that analytical strategies which use data directly coupling a specific agent (inhibitor, stimulator, signal, etc.) to a change in a final developmental phenotype usually have to contend with relative ignorance of context. By analogy, one understands a specific key, and a keyhole, but does not

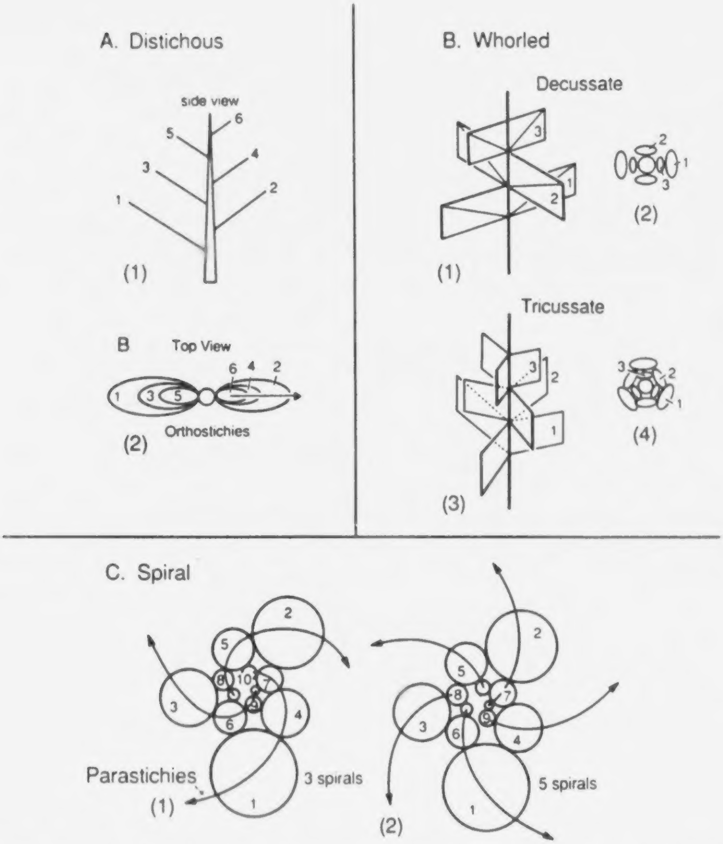


FIG. 5. The three major phyllotactic patterns. Leaves are numbered in order of their initiation. A. Distichous is a zig-zag pattern in a plane. In top view, the pattern has two ranks; each is called an orthostichy. B. Whorled. Two or more organs are initiated together at a node, successive whorls "nest" with new organs bisecting the angle between pairs of older organs. C. Spiral. The top view of a meristem is shown twice. In (1), three clockwise spirals, parastichies, pass through all the leaves. In (2), five counter clock-wise spirals pass through all the leaves. The phyllotaxis is called 3:5 in this case. The divergence angle between consecutive leaves is the Fibonacci angle, about 137.5°.

thereby automatically understand the workings of the lock. Such analyses often presume that a summing of many such couplings will explain the development. When the causal chain has an integration step, however, simple summing is inappropriate and interpretation can be obscure. An alternate strategy, advocated here, is to analyze from the phenotype through antecedent causes, thereby analyzing the activity of the "lock" in terms of its functional components, which often have differential character.

This approach will be illustrated in Part II, an analysis of the large scale patterns in plant development. The geometry of shoot behavior will be reduced to rules pertaining to tissue behavior. The tissue behavior can be traced to cytoskeletal phenomena, and finally to the genome.

THE INHERITANCE OF PATTERN IN PLANT SHOOTS

A widespread developmental phenotype is the regular arrangement of shoot structures on the axis of plants. The spiral, or helical, deployment of reproductive structures seen in sunflower heads and pine cones has fascinated observers since antiquity. These spectacular spiral examples should not overshadow the fact that virtually all shoot structures, vegetative or reproductive, are produced in a regular pattern of one sort or another (Schwabe, 1984). As a first step in seeking the mechanism of their production, it is necessary to characterize the patterns. Three major ones, described in Esau, 1977, are shown in Figure 5.

There are three major phyllotactic patterns

The simplest arrangement of organs is termed distichous (two ranked), or alternating, left and right, in a plane (Fig. 5A). This zig-zag pattern is typical of many monocots including grains such as corn. Distichy is found in the iris, the famous "Traveler's palm," etc. Ivy and pea are dicotyledonous examples.

The second category is called whorled (Fig. 5B). Here, more than one leaf, or organ, is produced at the same time and at the same height on the apical dome, ideally. Whorls of two opposite leaves, with the pairs successively rotated by 90°, form a decussate arrangement. This pattern is found in maple trees, snapdragons, and mint plants. Whorls of three, rotated successively by 60° ("tricussate"), are found in the oleander shrub. Many simple flowers have their floral organs in successive whorls: sepals, petals, stamens, carpels. There are whorls of three in the floral parts of the tulip and iris; whorls of five are found in many simple flowers of the succulent family, Crassulaceae.

The third category is the most famous and has obvious spiral features. We will deal only with the common Fibonacci spiral forms. Examples include oaks, many palms, willows, mustards, and in fact most plants. The spirals are termed "Fibonacci" in honor of a mathematician who is associated with a series of numbers used in characterizing the various spiral patterns. The series goes: 1, 1, 2, 3, 5, 8, 13, 21, etc. It is generated by adding two consecutive members to give the next member. In many spiral patterns, such as that of the florets in a sunflower head, the eye is caught by two sets of spirals of opposing sense (right *vs.* left handed). If all the spiral lines of one sense are counted, and compared with the number in the other set, it is typical to find the two numbers to be consecutive members of the Fibonacci series (Fig. 5C). Simple helical structures such as a pine cone will be low in the series, *e.g.*, 5:8. Sunflower heads may be high, such as 34:55.

When treated as a fraction, successive pairs of numbers approach the "Golden Ratio" which cuts a circle into two arcs of approximately 222.5° and 137.5° (about 62%, 38%). This ratio is special, or "Golden," because the whole circumference is to the big arc as the big arc is to the little arc. The developmental significance is that the small arc, about 137.5°, is the typical angle between successive organs in most spiral forms. This is called the divergence angle. Divergence is 180° in distichous patterns; it is 90° for pairs in a decussate pattern.

Phyllotactic patterns are variations on a single generative theme

All proposals for the basic mechanism of phyllotaxis have the form of logical loop. Special sites on the apical dome make leaves; recently formed leaves somehow determine the location of new leaf sites. The leaves' influence must act "inward" toward the center of the dome, against the general "outward" flow of all the cells on the dome. Any explanation of the production of the three patterns must involve such cyclic reciprocating activity.

There are reasons to believe that many important features of the phenomenology are shared in the three cases. Most obviously, the typical product of the activity, a leaf, has the same bilaterally symmetrical features regardless of the phyllotaxis. Further, in some cases the same plant can often shift from one pattern to another. For example, *Eucalyptus globulus* shifts from decussate to spiral after the tree reaches a certain state of maturation; similarly, ivy shifts from distichous to spiral (Rogler and Hackett, 1975). Upon flowering, many plants shift phyllotaxis to make a flower which is whorled. For example, the mustard family typically has spiral phyllotaxis in the vegetative form. The flowers, however, have 4 petals in a whorl. The iris shoot is distichous; the flowers have striking three-fold whorled symmetry.

Additional evidence for shared causation among phyllotactic systems comes from the work of Snow and Snow (1935). A shallow diagonal cut across the apical dome of *Epilobium*, a decussate plant, produces two half shoots each with spiral phyllotaxis. It is clear that the same genome can code for a variety of patterns. In light of these experiments, which brought on spirality surgically, it seems likely that the same state of gene activity can produce more than one pattern. Probably the shallow cut in *Epilobium* disturbed physical boundary conditions, rather than changed states of developmental gene activation, to shift the pattern. One thus expects that the three major patterns are variations on the same generative scheme.

Phyllotactic patterns are two dimensional. It is assumed that the basic mechanism of their development is also. The search for a plausible mechanism will proceed in two steps. First, the behavior will be reduced to rules described at the level of surface growth and histology. The rules will then be reduced to plausible biophysical and cellular mechanisms.

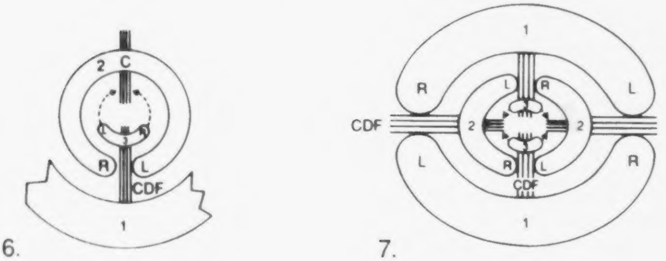
RULES FOR ORGAN BEHAVIOR IN PHYLLOTAXIS

A highly characteristic feature of a phyllotactic pattern is the divergence angle between successive leaves. As already noted, it is commonly assumed that a new leaf site is determined by the position and activity of recently formed leaves. Three empirical rules, describing developmental activity at the shoot meristem surface, can generate the dictichous and whorled patterns; related rules can account for spiral phyllotaxis.

In all cases, the leaf arises as a cresent-shaped ridge whose concave side faces the tip of the smooth apical dome. The primordium thus has a left tip, a central region, and a right tip. Rule one, for distichous phyllotaxis, is that early in its development, each leaf is involved in the production of a small group of parallel cell files running radially on the region of the dome just above the center of the leaf base. These will be called "central distal files" or CDF (Fig. 6). Rule two is that the ends of the ridge grow circumferentially, as pincers, on the dome, until encountering CDF from an older leaf. The resulting arc-shaped ridge is the base of the new leaf. Rule three is that a new leaf arises as a ridge when the left and right ends of a growing leaf base encounter central distal files. The tips of leaf base number n encounter the central files of leaf $(n - 1)$, the next older, to initiate leaf $(n + 1)$, as shown in Figure 6. The right tip of the old leaf base influences formation of the left tip of the new leaf, and the left, the right. The leaf-base growth occurs as a pincer's movement. This makes each growing leaf base serve as an angular bisector of the available circumference, defined as the circular arc length between central distal files, in this case 360°. The biophysics of converting a primordium to a leaf is addressed in Green (1986) and will not be covered here.

The same set of rules will also perpetuate the whorled pattern of primordia. There is only a trivial difference. In distichous plants, the pertinent right and left tips encountering the distal files come from the same leaf; in whorled plants they come from two different leaves (Fig. 7). This difference need have no substantive effect on the

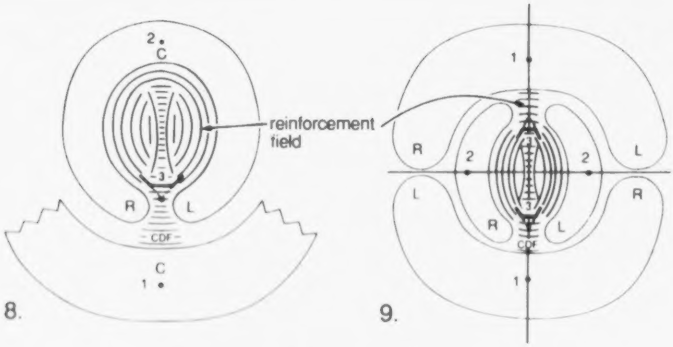
Phyllotaxis: Orthogonal Patterns



6. Distichous (Corn)

7. Whorled (Maple, mint)

Organ Level



8.

9.

Tissue Level Biophysics

FIGS. 6–9. Generative schemes for the orthogonal patterns. Leaves are numbered in order of their origin. These are top views, showing crescent-shaped leaf bases. Each leaf base has a central portion (C), and left (L) and right (R) tips. Pincers-like growth is shown by small arrows. Prominent cell files lying inward to the central region of a leaf are central distal files (CDF). FIGS. 6, 7. At the organ level, three rules suffice to perpetuate the patterns. a. New leaves grow as pincers until meeting CDF. b. Leaves produce their own CDF. c. A new leaf starts interior to where the pincers encounter CDF and grows in the opposite sense. In these cases the leaf-base growth bisects the available circumference (*i.e.*, that between CDF). Leaf initiation is at a site on the bisector of the angle made by the growth arrows of the arriving leaf-base tips. FIGS. 8, 9. Reinforcement patterns (lines) on apical domes in relation to cyclic initiation of leaves. Each leaf has associated with it a reinforcement field with lines of cellulosic reinforcement roughly tangential to the inner face of the leaf base. FIG. 8. A distichous apex. Below center, a small portion of the field from leaf 1, CDF, plus nearby portions of the left and right parts of the field of leaf 2, combine to give a three-membered U-shaped reinforcement pattern at the site for leaf 3. This is a leaf-site field. Presumably the buckling that forms the new leaf is fostered by the relatively high curvature at this site. Buckling occurs along the reinforcement lines (lines of least resistance) and smooths the three-membered reinforcement irregularity into a smooth arc, the new leaf. This can explain how the angle between R and L tips of leaf 2 is bisected by the new leaf 3 as in Figure 6. Subsequent pincers-like growth of leaf 3 is based on the buckling continuing along lines of reinforcement. FIG. 9. Arguments identical to those for the distichous dome suffice to perpetuate a whorled (decussate) pattern. R and L fields now come from different leaves.

mechanism. In both cases the scheme results in the repeated bisection of the available circumference by the new leaf, to give the divergence. While this available circumference is 360° in distichous phyllotaxis, it is 180° or less in whorled phyllotaxis. In both distichous and whorled forms, the leaves lie on orthostichies. These are straight radial lines that later become vertical. Along these lines there is continuity of central distal files. The files pass up one side and down the other side of a leaf (along the mid-rib region), and then pass through a gap between the leaves before passing over a subsequent leaf.

The corresponding generative algorithm for plants with Fibonacci spiral patterns is less obvious. Unlike the whorled forms, the two leaves which are lateral neighbors to a future leaf site are not at the same height on the dome (same distance from its center). Unlike whorled and distichous, no obvious bisection of an angle is carried out. Rather, a new leaf forms with its center at or near the *Golden Section* of the angle between the two neighboring leaves. The size of this angle is a function of the rank of the meristem in the Fibonacci series. If low, *e.g.*, 3:5, the angle is about 84.5°; in higher forms, *e.g.*, 8:13, the angle will be smaller. In all cases the Golden Section of the angle is taken, dividing it into arcs which are about 62% and 38% of the original. The new leaf forms on this off-center dividing line, closer to the older neighboring leaf.

A suitable generative algorithm, obviously provisional, is that two adjacent leaves "cause" a new leaf to arise at the Golden Section of the angle between them. Thus in Figure 10, leaves 2 and 4 "section" the angle between them to initiate leaf 7 at about 32° from leaf 2. This new leaf's base will expand laterally as a pincer's movement, as in the scheme for distichous and whorled. The radial distance of the new leaf from the center of the dome is predicted by its plastochron age difference from the two neighbors. In 3:5 phyllotaxis the plastochron ratio (fractional radial displacement per cycle) is about 1.2. Hence, the new leaf will arise at $(1/1.2)^3$ of the radial distance to the younger leaf, $(1/1.2)^5$ of the radial distance to the older.

If the production of the three phyllotactic patterns is a "variation on a theme," then one must show how similar phenomenology can give a *Bisection* of an angle when the lateral leaf structures are at the same height on the dome and a *Golden Section* when two lateral leaf structures are at different heights. To address the question of how older leaf primordia could "section" an angle in any fashion, one must have more detailed knowledge of the biophysics of the leaf primordium and the apical dome.

Primordium formation relates to patterns of reinforcement direction

The specific "sectioning" of an angle by a small number of older leaves is manifest as a local buckling of the dome surface at the particular angle. This initiates a leaf. The analysis here will characterize the buckling and then explore how the older leaves could influence its angular location.

Over-all, the dome surface is reinforced by cellulose microfibrils (Green, 1985). The lines of reinforcement, passing over many cells, are roughly concentric on the dome, *i.e.*, the radius of curvature of the reinforcement lines generally increases with distance from the dome center. A leaf primordium arises when a crescent region of this surface buckles, forming a crease on the inner side of the fold. At the site of buckling the reinforcement lines appear to be arcs, facing the dome center, which are somewhat more curved than expected at that distance from the dome center. Such regions are called *leaf-site fields* (Fig. 8).

The immediate cause of buckling is thought to be that the peripheral regions of the growing dome surface exert pressure on the more central regions; stresses there are relieved when local buckling occurs. The crease of the buckling occurs along the reinforcement lines, the path of least resistance. The new fold has the appropriate curved bilateral symmetry of a new leaf and faces the center of the dome. Subsequent pincer-like growth of the leaf base also follows the reinforcement lines.

With this view of the actual bulging process, the remaining issue is to find how action of the older neighboring leaves could set up the proper reinforcement irregularity at the appropriate location for a future primordium. Such a bilaterally symmetrical site must have a highly curved reinforcement pattern with its concave side facing the center of the dome.

New leaf-site fields arise as composites of parts of older fields

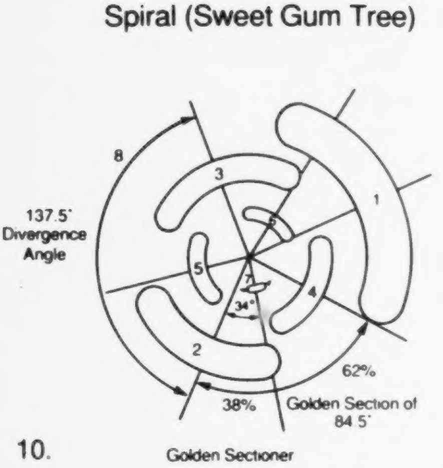
It has been observed that established leaves have multicellular fields of aligned reinforcement on the dome surface between the leaf and the center of the dome (Green, 1985). These fields arise, apparently, as a cytoskeletal response to the very rapid lateral growth of the primordium when it first formed. The leaf stretches the nearby dome, and the dome is thought to respond by modifying its structure to make a field. A leaf field is modeled by drawing circular arcs of constant radius distal to each leaf, using the distance from the leaf base to the dome center as the radius. Each field has a left, central, and right portion (Fig. 8). Once initiated, the curvature of reinforcement in an established leaf field slowly decreases as growth displaces the leaf from the center.

The proposed mechanism for leaf initiation is that if each older leaf has a reinforcement field interior to it, new leaf-site reinforcement patterns can arise by the *combining* of parts of older fields. While each older reinforcement field is itself of gentle curvature, parts of two or more established fields can combine, in an initially disjointed fashion, to give a leaf site field of sharper curvature. The disjointed character would disappear as buckling occurs and an arc-like ridge is formed.

The pattern for field combination to make leaf-sites is straightforward for distichous and whorled patterns. In the case of distichous phyllotaxy, a curved three-membered U configuration is formed at the leaf site (heavy bracket in Fig. 8). The base of the U comes from the central field of the leaf which is two cycles older than the leaf being formed. The left and right sides of the U come from the right and left fields, respectively, of a leaf one cycle older. Alignment in the left and right fields can be extrapolated to make a V. The new surface buckles along this V thereby "bisecting" the angle between the lateral reinforcement fields and, through subsequent pincers growth, also the available circumference. For leaves in whorls, the local situation is the same except that the two lateral sides of the U come from different leaves, not one leaf (Fig. 9).

A closely related process can operate in the spiral forms. Here, however, a Golden Section of an angle is taken and only two leaves are involved. Instead of a three-membered U or bracket configuration forming the leaf-site irregularity, a two-membered V pattern is formed directly. Both patterns can smooth into arcs of reinforcement which face the center of symmetry.

The interaction of reinforcement fields in spiral forms is modeled in planar diagrams. The alignment of the reinforcement fields in nature is known only approximately (Green, 1985). The most attractive explicit assumption is, as before, that the curvature at the base of each of the two older leaves is characteristic of each field. Many lines with this radius of curvature are drawn in front of two neighboring leaves, as in Figure 11. This is done by putting the compass point at the center of symmetry of the dome to draw the first arc at the leaf's base. Subsequent arcs are drawn by moving the compass point farther from



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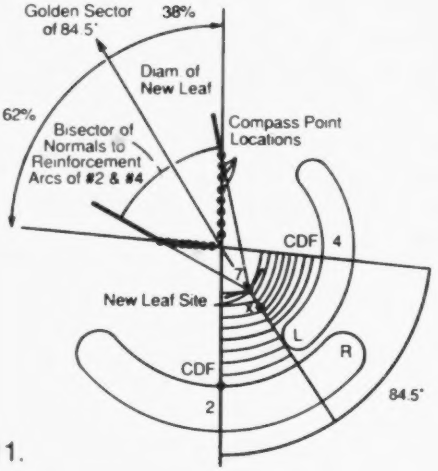

11.

FIG. 10. In spiral forms, successive leaves are at the Fibonacci angle which makes the Golden Section (38%–62%) of a circle. A new leaf, *e.g.*, 7, appears at the Golden Section of the "available circumference" (84.5°) between centers two leaves adjacent in space but not consecutive in age. The leaf then grows as a pincers, giving its base the typical bilateral symmetry.

FIG. 11. Interpretation of the reinforcement pattern on a dome with spiral phyllotaxis (*Ribes*: Green, 1985). Reinforcement lines are constructed, for each leaf, with constant curvature equal to that found at the leaf base. Successive lines are drawn as the compass point is moved along a diameter passing through the leaf and the dome center. The two fields abut along the diameter on which leaf 7 will form. Both fields are skew relative to the dome center. Nonetheless they combine to form a broad V-shaped reinforcement irregularity similar to the U-shaped one in other forms (heavy V). The V faces the dome center, approximately. Its alignment is also seen in the sharper V made by normals to the arcs. The angle of the bisector of this localized reinforcement irregularity approximates the angle which takes the Golden Section of the larger arc between leaves 2 and 4. The correspondence is exact at a point, X, peripheral to the leaf site. It is possible that the reinforcement pattern at the point X is the critical biophysical input to initiate a leaf. Repeated leaf initiation at the local Golden Section (*e.g.*, between leaves 2 and 4) should perpetuate a spiral pattern. The cyclic generation of crescent-like reinforcement irregularities is proposed as the common basis for phyllotactic patterns in general.

the leaf, along a diameter passing through the leaf center and dome center. One does not change the radius. The arcs drawn are not generally parallel. Because of the movement of the compass point away from the dome center, all but the first arcs are skew relative to the symmetry of the dome. This is true for both sets of arcs. Because of the skewness, the arcs intersect to make V-shaped reinforcement irregularities. The structure is locally bilaterally symmetrical, the concave side (bisector of the V) tending to face the center of symmetry of the dome (Fig. 11). Reinforcement irregularities of this sort have been seen at leaf sites in spiral forms (Green, 1985).

It is intriguing that, at the new leaf site, the V faces almost directly toward the center of symmetry. The error is about 3 degrees in 3:5 phyllotaxis. In a peripheral region below the leaf site, the V pattern faces the center of symmetry exactly (X in Fig. 11). It is suggested, as before, that the "sectioning" of the angle between the two leaves takes place by a physical buckling at a V-like junction of the two lateral fields, the V facing the center of the dome. Because the two fields are each skew on the dome, this bisection relative to the fields makes an approximate Golden Section of the angle between the two leaves (see Fig. 11).

Why is the explanation approximate? First, it might be necessary for the pertinent symmetry of buckling forces to exist in a region behind the future crease, rather than at the leaf site itself. If so, the explanation may be precise. Second, the model assumes strict circular arcs for the reinforcement fields; the real lines of reinforcement may be parabolic, elliptical, etc., in nature. This distinction could lead to a solution exactly at the leaf site. Finally, the model is constructed on a plane, whereas most domes are curved surfaces. It is hoped that ultimately this type of biophysical phenomenology will be able to explain the origin of a leaf site in terms of both its angle and its radial distance from the center of symmetry.

DISCUSSION

The development in spiral and non-spiral forms appears to be based in a common generative scheme. A reinforcement irregularity is formed through the recombination of reinforcement fields from recently formed organs. The field becomes a precursor to the new organ. The reinforcement irregularity has several important features: it is bilaterally symmetrical, faces the center of symmetry of the dome, and has differences on its upper and lower sides because only the concave (upper) side will buckle to form a crease. These features, seen on the dome, anticipate the major aspects of the organ to come. These features have their biophysical antecedents in the action of growing organs on the dome. In this way the logical loop that "dome structure makes leaves," and "leaves make dome structure," is completed.

The diversity of phyllotactic patterning can be initially reduced to an organ-level algorithm where activity of two or three leaves determines a new leaf site. In distichous and whorled forms the angle between two leaf base tips is bisected; in spiral forms the angle between two leaves is sectioned in the golden proportion, the new leaf appearing nearer the older member of the original two. It is postulated that in all cases the immediate morphogenetic function of a growing new primordium is the same: to produce a reinforcement field distal to itself, on the dome. In all patterns the combination of parts of several such fields produces a new, arc-reinforced, leaf-site field. The differences between patterns relate to initial conditions or secondary features of leaf base growth. These determine the geometry in which the cyclic combination occurs.

The genetic basis of the production of a reinforcement field apparently relates to cytoskeletal activity. Microtubules are thought to govern the orientation of the cellulose reinforcement (Gunning and Hardham, 1982; Lloyd, 1982). An attractive hypothesis for the generation of a reinforcement field on the dome is that the excessively rapid transverse stretch of dome tissue distal to the new primordium orients cell division, and also cellulose reinforcement, to run parallel to the lines of stretch.

The gene products which give the cytoskeletons this orientation capacity would therefore be responsible for the ability to carry out phyllotaxis in general. The gene products responsible for any specific type of phyllotaxis, *i.e.*, those that relate to boundary conditions and growth behavior of leaf bases, are more obscure. It is likely that their identification will require further refinement of the generative activity of apical domes. The hierarchical analysis is summarized in Table 1.

It is a major point of this article that developmental progressions, including cyclic ones such as phyllotaxis, can be interpreted as the consequence of the following of rules which have differential character. That is, the rules couple a response to a condition. Once identified, the rules can be investigated to find their cytological and nuclear basis. A second major point is that such rules, and their ultimate basis in the genome, may often be more readily ascer-

TABLE 1. *Summary of analysis: the major patterns.*

Distichous	Whorled	Spiral
	Decussate Tricussate	
1. New leaf arises with a divergence equal to the *Bisector* of a whole circumference.	New leaf arises on the *Bisector* between adjacent leaves.	New leaf arises on the *Golden Section* between two older leaves.
↓		↓
2. Leaves bisect the "available circumference" (*i.e.*, the arc between central distal files).		
↓		
Orthostichies		Parastichies
↓		↓

Organ rules

Basis of bisection:
- a. Leaves make central distal files
- b. Leaf bases grow as pincers and stop at central files
- c. New leaves arise where pincers stop

↓

Tissue rules

3. Leaf arises as localized crescent-shaped *buckling*, along special pre-established cellulose reinforcement lines (lines of least resistance). The reinforcement irregularity is a V or U with concave side facing the center of shoot symmetry.

↓

4. The various phyllotaxies regenerate these irregularities through the juxtaposition of *parts* of older single reinforcement fields. The mode of cyclic combination determined the particular phyllotaxy.

↓

5. Single reinforcement fields arise on the dome, each field distal to a recently formed leaf.

↓

Cell rules

6. Reinforcement fields arise in cells on the dome apparently in response to rapid circumferential stretch by nearby leaf base growth.

↓

7. This traces to a presumed cytoskeletal response.

↓

8. Microtubules and associated proteins.

↓

9. RNA.

↓

10. DNA. Other genes pertain to secondary factors bearing on level #4 to generate particular patterns.

tained if one analyzes from the phenotype toward the gene.

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Limb Development and Regeneration[1]

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SYNOPSIS. Experiments on developing and regenerating vertebrate limbs have led to the idea that pattern formation and growth control are causally linked. The mechanism by which position-specific growth occurs is termed intercalation, and evidence is presented that implicates intercalation in the initiation, maintenance and cessation of growth during limb formation. We conclude that among the variety of cell types present in limbs, only fibroblasts have been shown to possess the positional information necessary for intercalation. Hence we propose that the limb pattern is generated by intercalation between fibroblasts to give rise to a connective tissue scaffold, which in turn dictates the positioning and morphogenesis of all of the differentiated cell types of the limb. Finally, we review evidence that regenerative failure among higher vertebrates is linked to defects in the intrinsic cellular mechanisms of growth control (intercalation) and conclude that progress towards the goal of stimulating regenerative limb outgrowth in non-regenerating vertebrates will be contingent upon a better understanding of these intrinsic mechanisms.

INTRODUCTION

Developmental biology is the study of the processes of growth and change in organisms. The fundamental features of these processes have persisted through countless years of evolution such that within a given species, individuals with the same highly ordered arrangement of differentiated cell types are repeatedly produced. At the same time, variation in specific aspects of these processes have allowed for the emergence of a vast and diverse array of biological patterns. Given the breadth and complexity of developmental biology, it should not surprise us that it is studied from many different perspectives, involving numerous and diverse approaches. All such approaches will ultimately become integrated into an understanding of how cells, as the units of development, interact with one another and with their environment to produce the characteristic patterns of the organism and its various parts. This understanding will involve knowledge about what activities the cells engage in at any point in time, what stimuli the cells respond to, and how the cells utilize genetic information to direct these activities.

The vertebrate limb has proven to be a valuable model system for gaining insight into the cellular mechanisms of growth and pattern formation. In our efforts to understand the development and regeneration of vertebrate limbs, we began with experiments to deduce which of the many activities available to cells are most likely to be involved in limb patterning. More recently we have used these deductions to guide our studies at the level of individual cells. In turn our knowledge of cell behaviors in limb patterning will guide studies of the molecules involved in pattern formation. This essay represents our attempt to integrate the various levels of our current understanding of development and regeneration of the vertebrate limb.

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OVERVIEW

The task we have set for ourselves is to come to an understanding of limb outgrowth in development and regeneration, and it is appropriate to begin with a description of the problem at hand. Fully grown limbs are a complex of many differentiated cell types, the most obvious of which are epidermis, dermis, muscle, bone and/or cartilage, nerves, blood vessels and loose connective tissue. These tissues are arranged in a well defined pattern that we recognize as changing from proximal to distal levels (*e.g.*, upper arm with humerus, lower arm with radius and ulna, wrist with carpals, and hand with metacarpals and phalanges) and from one part of the circumference to another (*e.g.*, anterior radius, posterior ulna, anterior digit 1, posterior digit 5, dorsal extensor muscles, and ventral flexor muscles). Although the question of how each cell type differentiates during development is of interest, we have focussed on a rather different issue, namely that of how patterning is controlled within cells with the same differentiated character. The fact that cells with a particular differentiated phenotype are non-equivalent in terms of positional information (Lewis and Wolpert, 1976) can be simply illustrated by the following example. Skin at all positions around the limb circumference is composed of the same types of differentiated cells; however anterior and posterior skin are non-equivalent positionally in that homotopic grafting of skin strips results in normal limb regeneration following amputation, but heterotopic grafting results in the formation of supernumerary structures (discussed in detail below). Thus an understanding of how the pattern of structures develops is dependent upon more than a knowledge of how skin cells differentiate, and can only be achieved from an understanding of the nature and mechanisms of interactions between positionally non-equivalent cells.

At the earliest times in limb outgrowth, both developing and regenerating limbs consist of a uniform-looking accumulation of proliferating mesenchymal cells (blastema) overlain by a thickened epidermis (Fig. 1). In developing limbs, the mesenchyme is derived from the lateral plate mesoderm (somatopleure) of the flank (Harrison, 1918). In regenerating limbs, the mesenchyme is derived from the local tissues of the stump, which dedifferentiate, accumulate beneath the newly healed wound epithelium, and proliferate (see Wallace, 1981). The information for constructing the limb pattern in both cases resides in the mesodermally derived blastema (Harrison, 1918; Tschumi, 1957; Kieny, 1960; Stocum and Dearlove, 1972; Saunders and Reuss, 1974). The limb pattern is laid down in a specific proximal-distal (Saunders, 1948; Tschumi, 1957; Stocum and Dearlove, 1972; Summerbell, 1974) and anterior-posterior (Shubin and Alberch, 1986) sequence.

Our ability to investigate the mechanisms underlying limb outgrowth depends on the phenomenon of pattern regulation exhibited by developing and regenerating limbs. If slices along the proximal-distal axis are removed, the final limb is nevertheless complete (Iten and Bryant, 1975; Stocum, 1975; Kieny and Pautou, 1977; Summerbell, 1977; Pescitelli and Stocum, 1980; Maden, 1981). If tissues from one part of the bud circumference are brought into contact with those from a distant part, the cells respond by forming additional or supernumerary limb structures (see Maden *et al.*, 1983; Javois, 1984; Muneoka and Bryant, 1984*a*). Of particular importance is the fact that urodelean amphibians exhibit pattern regulation in response to such deletions and tissue rearrangements throughout life, an ability unique among the vertebrates. As a consequence, interactions between developing and regenerating tissues can be analyzed experimentally (Muneoka and Bryant, 1982; 1984*c*). Such studies have demonstrated that the basic mechanisms involved in limb outgrowth during development are the same as those involved in limb regeneration. As discussed in more detail below, we have caused the formation of limbs of normal external and internal anatomy that are made up of equal numbers of cells from developing and regenerating tissues. Thus the mechanisms by which cells interact to form limbs are common to both development and regeneration, and the problem of understanding limb outgrowth in these two different situations has become in many respects a unitary one.

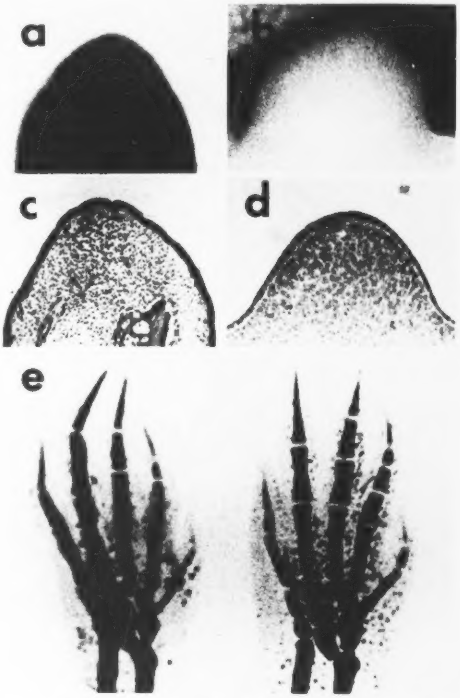

FIG. 1. Views of developing and regenerating limbs. (a) External view of a medium bud blastema on a newt limb amputated through the upper arm. (b) External view of mouse limb bud at 11 days of gestation. (c) A light micrograph of a longitudinal section through a medium bud blastema from a newt forelimb. H&E staining. (From Iten and Bryant, 1973.) (d) A light micrograph of a longitudinal section through the limb bud of a 10 day mouse embryo. Mallory's triple stain. (e) Whole mount skeletal preparations of an original axolotl limb (right) and its regenerate (left).

MODELS

Webster defines a model in the following way: "A description or analogy used to help visualize something that cannot be directly observed." In other words, a model of limb outgrowth is a tool which helps us to visualize the whole process at a time when all the facts are not known. Once we have a vision of the process, we can begin to construct hypotheses to test parts of this vision or model. Hence, the way that we think about the problem or the nature of our vision of it will determine which experiments we perform, which in turn determines the type of new information that will become available. When the object of our modeling is complex, and the data base is relatively small, it is possible to weave the available facts into more than one internally consistent model. In fact, the coexistence of several views of how an unknown process works is important for continued progress toward understanding that process. As with any tool, a model works best if it is easy for the operator to use. For example, a model couched in terms of interacting chemicals helps some people to visualize a particular process best; for others, more abstract mathematical models make the most sense. However, it is important to recognize that a model can only continue to be useful as a tool if it either continues to be internally consistent as new data emerge or if it changes to accommodate new facts.

The model that has facilitated our investigation of limb development and regeneration in vertebrates is the polar coordinate model (PCM), originally proposed in 1976 (French *et al.*, 1976; Bryant *et al.*, 1981). In this essay, we will present information regarding what is known about the growth of vertebrate limbs in the context of this particular model. It is our intention to provide an internally consistent framework within which to assimilate the specific facts to be presented, without trying to

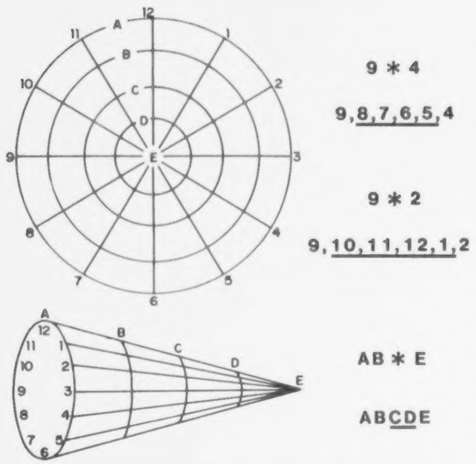

FIG. 2. The polar coordinate model (PCM). At left, polar coordinates of positional information in a limb, as viewed from the distal tip (upper) and from the side (lower). Each cell is assumed to possess information specifying its position around the limb circumference (1–12) and along the proximal-distal axis (A–E). Positions 12 and 0 are identical, thus the sequence of circumferential values is continuous. (From Bryant *et al.*, 1981). At right, intercalation of missing circumferential and proximal-distal positional values. When normally nonadjacent values come into contact (*) the missing values are restored by growth and insertion of the intermediate values (underlined). Circumferential intercalation generates confronted values by the shorter, rather than the longer, of the two possible routes (the "shortest intercalation rule").

convince or persuade the reader that this or any other particular model is "right" or "wrong."

The polar coordinate model

In the decade that has passed since its formulation, the PCM has been a useful guide to new experiments concerning limb outgrowth. During this time, parts of the model have remained intact as new data emerged, and parts have changed. The PCM is a formal model that presents a view of limb outgrowth based on hypothesized properties and behaviors of limb cells. The model was originally developed to account for the growth and regulation of three very different structures, in different states of differentiation, and in phylogenetically distant organisms: the imaginal discs of *Drosophila*, the regenerating legs of cockroaches, and the regenerating and developing limbs of amphibians. In this essay, we will only discuss its application to vertebrate limbs. Central to the PCM is the idea that growth and patterning are causally linked, and that cell-cell interactions are fundamental to both processes. Cells are proposed to possess information about their position along the proximal-distal axis, and around the circumference of the limb (positional information) (Fig. 2). In addition, it is proposed that these cells have the property of position-specific growth, referred to as intercalation. Intercalary interactions occur when cells with normally non-adjacent positional values come into contact, either as a result of rearrangements during development or wound healing, or as a result of grafting, and they result in cell division. Division continues within the system until all positional disparities have been eliminated by the intercalation of cells with appropriate intervening positional values. In terms of the circumferential sequence of positional values, where two possible routes of intercalation exist, intercalation is thought to proceed via the shorter of the two. These simple principles of the PCM account for limb outgrowth in development and regeneration, for regulation of the pattern following the deletion of parts, for the production of supernumerary limbs, and for the regulation of growth and final size. In the sections that follow, we see how these properties of limbs can be visualized in terms of the PCM.

POSITIONAL INFORMATION AND INTERCALATION

A fundamental concept of the PCM is that limb cells possess positional information. The most direct test of this concept is to change the relative positions of cells within the limb. If the repositioned cells bring about some change in their new neighbors or *vice versa*, we conclude that limb cells possess positional information and that they are capable of responding to neighbors with different positional information. The fact that these changes do occur in response to positional disparities created by wound healing or grafting indicates that limb cells do in fact possess positional information. Although the actual molecules involved in specifying positional information in limbs are at present unknown, such information clearly exists along the proximal-distal axis and around the limb circumference (Bryant *et al.*, 1981). We also know that the information present in small groups of cells can evoke a dramatic positional response (Tank, 1981; Tickle, 1981; Rollman-Dinsmore and Bryant, 1982) and that positional information is stable within cells for several days in culture (Honig, 1983; Carlson, 1984*b*). Although there are multiple hypotheses as to the physical-chemical nature of positional information, as is made clear below, we favor the view that it is encoded on the surface of individual cells.

The other major premise of the PCM is that some cells of the limb are capable of intercalation in order to eliminate positional disparities. The most direct evidence for the involvement of intercalation in limb outgrowth comes from experiments in which a positional disparity is created experimentally. For example, if limb buds or blastemas are grafted contralaterally so as to maintain the normal dorsal and ventral orientations, positional disparities are created where anterior cells of the host limb contact posterior cells of the graft and *vice versa* (Fig. 3; see Muneoka and Bryant, 1984*a*). At the sites of such positional value confrontations, growth of additional (supernumerary) limbs occurs. In terms of the PCM such supernumerary limbs are a product of intercalation to eliminate positional disparities (French *et al.*, 1976). The PCM makes no specific predictions about the origin of the cells forming supernumerary structures resulting from intercalary interactions. For example, it is possible that at the confrontation described above, only cells on one side of the disparity will respond by growth to form the new outgrowth. Should this occur, then it would be difficult to distinguish between models based on intercalation, and those based on the existence of separate populations of signalling cells that do not contribute, and responding cells that contribute but do not signal (*e.g.*, the ZPA model for the anterior-posterior pattern of the chick limb, Tickle *et al.*, 1975; see Fig. 4d). If, on the other hand, the outgrowth is derived from cells on both sides of the disparity, then intercalation as a mechanism is clearly supported, and at the same time, signalling mechanisms are made unlikely (Bryant and Muneoka, 1986). The cellular contribution to supernumerary limbs in amphibians and chicks has been analyzed by making use of cell markers (ploidy differences in axolotls, see Fig. 4a, c; nuclear staining differences between two species of *Xenopus*; and nuclear staining differences between chick and quail). The results show that when supernumerary limbs are formed in developing or in regenerating limbs, they are in fact composed of cells from each side of the confrontation (Thoms and Fallon, 1980; Carlson, 1984*a*; Maden and Mustafa, 1984; Muneoka and Bryant, 1984*b*; Javois and Iten, 1986; Muneoka and Murad, 1987). In the developing chick limb there is an undercontribution by posterior cells and a corresponding overcontribution by anterior cells that has been cited as evidence for a signalling-responding mechanism (Summerbell, 1981). Although the posterior region does not contribute half the cells to supernumerary limbs, it does form as much as one-third of the extra structures (Javois and Iten, 1986), a behavior that is inconsistent with it being comprised of signalling, but not responding cells. In developing and regenerating amphibian limbs, the cellular contribution

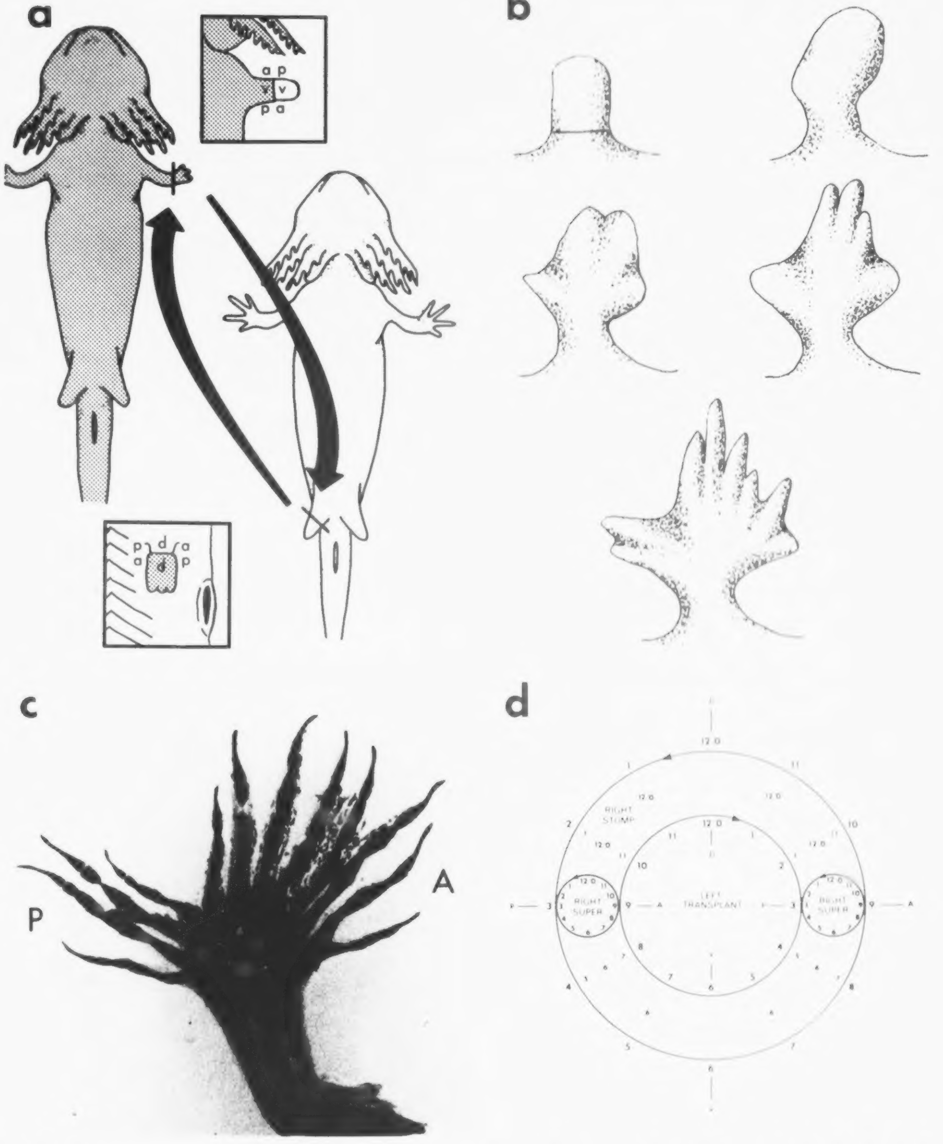

FIG. 3. **Supernumerary limb formation. (a) Diagram** of a grafting procedure that results in the formation **of supernumerary limbs in the axolotl (ventral view).** In this example the grafts are made between regenerating forelimb blastemas (palette stage) and developing hindlimb buds, and between triploid (dark) and diploid (light) animals. Equivalent results are obtained from similar blastema-blastema and limb bud-limb bud grafts. Grafts are exchanged between left and right limbs (contralateral) and oriented so as to maintain the correct dorsal-ventral orientations but to appose anterior and posterior tissues (upper insert, ventral view of a right hindlimb bud grafted onto a left forelimb blastema stump; lower insert, dorsal view of left forelimb blastema grafted onto a right hindlimb bud stump). a, anterior; d, dorsal; p, posterior; v, ventral. (From Muneoka and Bryant, 1984*c*.) (b) Stages in the appearance of supernumerary limbs in the axolotl, after grafting to confront anterior and posterior tissues. From left to right and upper to lower: 1, 4, 8, 12 and 18 days after grafting.

from anterior and posterior regions of both graft and host tissues is, on the average, half and half (Fig. 4b; Thoms and Fallon, 1980; Muneoka and Bryant, 1984*b*; Muneoka and Murad, 1987). Hence intercalation is clearly the most feasible explanation of the origin of supernumerary limbs in amphibians, and a reasonable explanation in chicks. In addition, the interactions between developing and regenerating limbs of the axolotl can be tested directly. In such experiments, positional disparities such as those described above, are made in which developing and regenerating limb cells face each other on opposite sides of the positional value confrontation (Fig. 3; Muneoka and Bryant, 1982, 1984*c*). The ensuing supernumerary outgrowths are well organized limbs, consisting of approximately equal numbers of cells from each side of the positional disparity (Fig. 4b). On this basis we conclude, as stated earlier, that the mechanisms by which developing and regenerating limbs grow out are the same. Further, we infer that intercalation is a mechanism that is common to both.

PATTERN FORMING CELLS

In order for the PCM to provide an adequate explanation for limb outgrowth, it is not necessary that all limb cells participate in pattern formation by intercalation. All that is required is that a population of cells have this ability, and that the remainder of the cells of the limb react secondarily to the positional information of the pattern forming population. Various lines of evidence suggest that this is indeed the case.

TABLE 1. *Distribution of positional information in cells and tissues of the limb.*

Tissue type	Cell type	Positional information
Epidermis	Epidermis	−
Dermis	Fibroblast	+
	Pigment	−
Nerve sheath	Schwann	−
Cartilage	Chondrocyte	−
Muscle	Myogenic	−
	Fibroblast	+

We will consider various cell populations and assess their role in limb outgrowth, particularly in terms of whether or not they are directly involved in organizing the limb pattern (Table 1).

The epidermis forms a discrete cell population on the surface of the outgrowth. The epidermis is necessary for limb outgrowth because if it is removed during outgrowth, truncated limbs are formed (Saunders, 1948; Tschumi, 1957; Stocum and Dearlove, 1972; Summerbell, 1974). In amphibians, if epidermis is removed, the remaining epidermis quickly heals over the denuded limb stump and is again capable of supporting further outgrowth of the limb. Hence, in order to demonstrate directly that the epidermis is necessary for limb outgrowth, limb bud mesoderm must be grafted to positions where it does not acquire a new epidermal covering (Tschumi, 1957; Stocum and Dearlove, 1972). In such circumstances, the mesoderm ceases growth and differentiates as distally truncated limb structures. In contrast to amphibians, the epidermis that heals over the experimentally denuded

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(From Bryant and Muneoka, 1986) (c) Skeletal preparation of an axolotl limb resulting from a graft of a left hindlimb bud onto a right forelimb blastema stump so as to appose anterior and posterior tissues (ventral view). Anterior (A) and posterior (P) supernumerary limbs formed which were composed of cells derived from both the forelimb stump and hindlimb graft as determined from diploid/triploid cell marker analysis. (From Muneoka and Bryant, 1984*c*) (d) The PCM interpretation of supernumerary limb formation. The inner circle represents a grafted limb bud or blastema and the outer circle the host stump. Intercalation between graft and host cells leads to the formation of two centers of outgrowth at the positions of maximal positional disparity (anterior and posterior). These centers will form supernumerary limbs with the predicted handedness as a result of further intercalary interactions (see Fig. 6e). Abbreviations as in (a). (From French *et al.*, 1976)

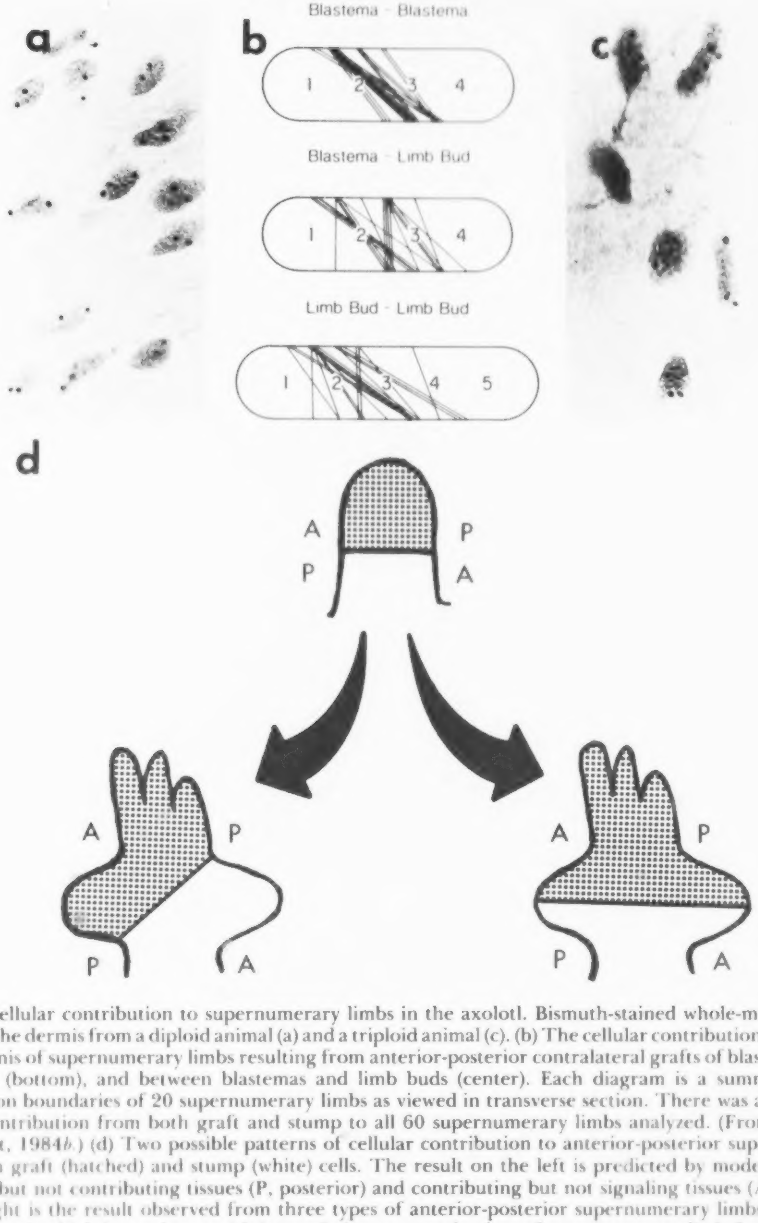

FIG. 4. Cellular contribution to supernumerary limbs in the axolotl. Bismuth-stained whole-mount preparations of the dermis from a diploid animal (a) and a triploid animal (c). (b) The cellular contribution boundaries in the dermis of supernumerary limbs resulting from anterior-posterior contralateral grafts of blastemas (top), limb buds (bottom), and between blastemas and limb buds (center). Each diagram is a summary of the contribution boundaries of 20 supernumerary limbs as viewed in transverse section. There was a substantial cellular contribution from both graft and stump to all 60 supernumerary limbs analyzed. (From Muneoka and Bryant, 1984*b*.) (d) Two possible patterns of cellular contribution to anterior-posterior supernumerary limbs from graft (hatched) and stump (white) cells. The result on the left is predicted by models involving signalling but not contributing tissues (P, posterior) and contributing but not signaling tissues (A, anterior). On the right is the result observed from three types of anterior-posterior supernumerary limbs in axolotls (see (b) above), which is consistent with intercalation as a mechanism of supernumerary limb formation.

chick limb bud is unable to support further outgrowth. This result has been interpreted to indicate that it is not the epidermis per se, but rather a thickened region of the epidermis along the distal margin of the limb bud (the apical ectodermal ridge, AER) that is required to support outgrowth. The AER does not reform when the epidermis heals, whereas a graft of a new AER does support outgrowth and grafts of an additional AER stimulate supernumerary outgrowths (see Saunders, 1977).

Despite the demonstrated necessity for the epidermis in limb outgrowth, the available evidence suggests that epidermis does not carry positional information. This conclusion is based on the observation that with heterochronic combinations of limb mesoderm and epidermis, development proceeds in accordance with the stage of development of the mesoderm alone (Rubin and Saunders, 1972). Similarly, prospective limb bud mesoderm grafted beneath flank epidermis in various orientations leads to the formation of limbs that are uninfluenced by the orientation of the epidermis and that conform to the orientation of the mesoderm (Saunders and Reuss, 1974). Finally, reorientation of epidermis relative to other limb tissues does not stimulate formation of supernumerary structures that, by definition, would arise if the epidermis did possess positional information (Carlson, 1975). Although it is not known exactly how the epidermis acts during limb outgrowth, it clearly is important in creating or maintaining an appropriate environment for the pattern forming population to carry out their program of activities.

The remainder of the limb bud or blastema consists of cells of mesodermal origin. In developing chicks, it has been shown that the muscle precursor cells are a distinct population derived from the somites, whereas the remainder of the limb mesoderm is derived from the somatopleure (see Gumpel-Pinot, 1984). The muscle precursor cells migrate into the limb bud, but they are not present at the tip of the limb where new pattern elements are being elaborated (Newman *et al.*, 1981). If muscle precursor cells from a different region of the body axis are grafted so that they invade the developing limbs, the muscles that form are characteristic of the limb mesoderm, not of the normal fate of the muscle precursor cells (Chevallier *et al.*, 1977). Similar results have also been reported for the patterning of the musculature of the head (Noden, 1986). In addition, if muscle precursor cells are prevented from migrating into the limb outgrowth, its overall pattern is nevertheless normal, though lacking muscles (Christ *et al.*, 1977). Although comparable evidence concerning the origin of muscles is not available for developing amphibian limbs, it has been shown that during regeneration muscle will only form in the outgrowth if viable muscle cells were present in the stump. Here too, muscle cells appear to form a discrete lineage, and regeneration of normally patterned, though muscleless limbs can occur (Namenwirth, 1974; Dunis and Namenwirth, 1977; Lheureux, 1983). Hence myoblasts are unlikely to be involved directly in the generation of the limb pattern.

In regenerating limbs, the mesodermal blastema is made up of cells that originated in the tissues of the stump. Although blastema cells have the morphological characteristics of a fairly uniform population (Salpeter and Singer, 1960), their separate origins present the opportunity to inquire about the role of cells of different origins in limb outgrowth. This is in contrast to the presumed unitary origin of the developing limb bud mesoderm (non-muscle) from the somatopleure. One approach that has proved important in assessing which cell population(s) is directly involved in pattern formation, has been to alter the position of a particular tissue in the stump prior to amputation. For example, when a skeletal element is rotated in the stump (Carlson, 1975), or if an extra skeletal element is added (Goss, 1956) there are no consequences for the regenerated pattern, which is normal. Similarly, nerve sheaths implanted into new locations in the stump do not alter the pattern of outgrowth

(Muneoka, unpublished). Hence, we conclude that neither the skeletal tissue nor the nerve sheath cells of the limb are directly involved in patterning. Dramatically different results are obtained however, when either skin or muscle are reoriented in the stump (Carlson, 1974*a*, 1975; Tank, 1981; Rollman-Dinsmore and Bryant, 1982). Such grafts lead to the formation of supernumerary structures during regeneration, even when the grafts are very small. In the case of skin, which consists of epidermis and dermis, the epidermis does not possess positional information (see above), and thus we conclude that the cell population involved in patterning is in the dermis. The dermis consists primarily of fibroblasts and pigment cells (Holder and Glade, 1984), and since pattern regulation occurs in limbs without pigment cells (see Wallace and Wallace, 1973), it is most likely that the pattern forming cells are the fibroblasts. In terms of muscle grafts, we have already presented evidence to rule out the myogenic cells as being involved in patterning, which again leaves the fibroblasts of the muscle connective tissue as the cell population involved in pattern formation.

Direct evidence concerning the relative roles of different tissues in limb outgrowth has been obtained using marked grafts to quantify cellular contribution. Earlier descriptive studies by Chalkley (1954, 1956) suggested that all tissues transected by amputation undergo a process of dedifferentiation to yield cells that enter the blastema. Based on these results, it was assumed that all tissues contribute cells in proportion to their availability in the stump. In recent quantitative studies utilizing the ploidy marker in axolotls, it was found that neither cells derived from skeletal tissue (Muneoka *et al.*, 1986*a*), nor those derived from nerve sheaths (Muneoka, unpublished) contribute extensively to the blastema. In fact, cells from these two tissues were underrepresented in the blastema compared to their relative availability in the stump. On the other hand, it was found that cells of the dermis that represent 19% of all stump cells, make up on the average 43% of the blastema. Therefore, the cells (fibroblasts) that have been identified as possessing positional information and the ability to intercalate, are the same cells that are overrepresented in the early blastema compared to their availability in the stump. Furthermore, if fibroblasts of dermal origin make up about half of the early outgrowth, the bulk of the other half conceivably could come from other connective tissue fibroblasts of the stump, since we know that the dermis makes up about 50% of all connective tissue cells (excluding cartilage and bone) (Tank and Holder, 1979; Muneoka *et al.*, 1986*a*). In conclusion, we infer from the various pieces of evidence outlined above, that positional information in the limb is a property of fibroblasts, and that it is these cells that undergo intercalation in response to positional disparities. We propose that these cells build a patterned template, and that other cells (*e.g.*, myoblasts, nerves, endothelium) use this template to construct the complete limb pattern.

A VIEW OF LIMB OUTGROWTH

In the sections above, we have presented evidence that some limb cells carry positional information, and that they exhibit intercalation. In developing limbs, the cells with these properties are the non-myogenic mesoderm cells derived from the somatopleure. In regenerating limbs, the non-myogenic cell population has been further dissected to indicate that the pattern forming cells are the fibroblasts of the dermis and loose connective tissue. In this section we present our ideas about how these cells regulate limb outgrowth. The initiation of limb outgrowth will be considered in two different situations: in regeneration, where initiation begins at an amputation plane, and in development. Since we know the least about the latter, we will discuss the initiation of limb outgrowth in regenerating limbs first.

Regenerating limbs

Following amputation, all of the mesodermal tissues of the limb are transected. The epidermis at the wound margins quickly (within 12 hr) migrates over the stump, and then thickens into an apical cap with the outgrowth-permitting properties described earlier. Beneath the apical cap, the tissues in the vicinity of the wound lose their differentiated appearance and release cells which appear unspecialized. This process is known as dedifferentiation. During this process, cells display blastema specific antigens (Kintner and Brockes, 1985). There has been general agreement for some time that the blastema is formed from these dedifferentiated cells, and that it is these cells that proliferate and subsequently redifferentiate to give rise to the regenerated portion of the limb. Our basic hypothesis is that positional disparities created by cell migration after wounding drive limb outgrowth by the process of intercalation. Indirect evidence for such migrations has been obtained from two different studies. In one, limb stumps were created in which half of the stump was diploid, and half was triploid (Muneoka *et al.*, 1985; Tank *et al.*, 1985). After regeneration, it was found that although 75% of the cells of a particular ploidy remained on their side of origin in the stump, 25% were found on the opposite side of the regenerated limb. In the other study, small strips of triploid skin were added to the circumference of diploid limbs, and in the regenerate, triploid cells were found to be scattered throughout (Rollman-Dinsmore and Bryant, 1984). These results showed that cellular displacement is occurring sometime during regeneration. We suspected that the displacement was occurring during wound healing, since in limbs that grow out from an experimentally created positional disparity (in which there is no amputation plane to heal), cells that start out on opposite sides of the confrontation, maintain clear boundaries in the outgrowth. We have recently demonstrated directly that dermal fibroblasts, that are situated around the periphery of the limb at the time of amputation, migrate centripetally beneath the wound epidermis, beginning at about 5 days after amputation (Fig. 5; Gardiner *et al.*, 1986). Hence, the cells that possess positional information, and that are capable of intercalation, migrate in such a way that they will come into contact both with cells from distant parts of the limb circumference (other migrating dermal cells) and with more centrally located cells (cells of the loose connective tissue within the stump), thereby creating the positional disparities necessary for outgrowth (Fig. 6a, b). Furthermore, studies of the onset of mitosis in the stump in response to amputation show a significant rise in cell division at about 5 days (Kelly and Tassava, 1973; Tassava *et al.*, 1974; Maden, 1978), which is the time at which cells begin to migrate and as a consequence begin to acquire new neighbors.

Developing limbs

The initiation of outgrowth in developing limbs is not well understood. By analogy to what is known for regeneration and supernumerary limb formation, we hypothesize that outgrowth of the developing limb is stimulated by positional disparities, but direct evidence for this is presently unavailable. There are, however, early events in the development of the limb that could stimulate cell migration and subsequent intercalation. Borgens (1984) has suggested that the larval epidermis becomes "leaky" to ions in the region of the prospective limb, and that the current flow that is thereby generated causes the aggregation of mesenchyme cells to form a limb bud. In support of this view, currents with strengths which may be sufficient to affect cell migration have been detected not only

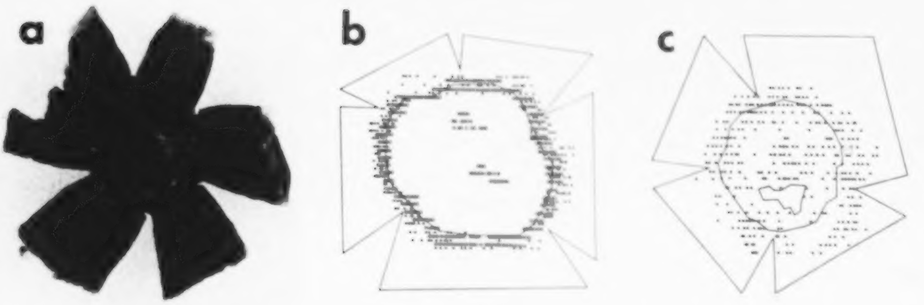

FIG. 5. **Migration of dermal cells across the wound surface in axolotls. (a) Whole mount preparation of the under surface of the wound epithelium (central area) and more proximal full thickness skin 10 days postamputation. (b) and (c) Computer-assisted plots of the distribution of dermal cells under the wound epidermis at 5 days after amputation (b) and at 10 days (c). The centrally located cells in (b) are cells of stump origin. The small outlined area in (c) is cell free. (From Gardiner *et al.*, 1986.)**

following amputation prior to regeneration, but also at the sites of prospective limbs in axolotls and frogs (see Borgens, 1984). Furthermore, ultrastructural studies in *Xenopus* have shown that in the region of the presumptive bud, the extracellular matrix of the basement lamella becomes disorganized, mesenchymal cells invade the disorganized region, and gap junctions develop between them (Fig. 6c, d; Kelley and Bluemink, 1974). These observations suggest that mesodermal cells are migrating at the time of limb bud initiation, that they have the opportunity to acquire new cellular neighbors, and therefore the opportunity to create the positional disparities that we propose are necessary to drive the outgrowth. Such an early migratory event could also account for the apparent sequential establishment of the different axes of the limb field (anterior-posterior before dorsal-ventral) as demonstrated by grafting experiments (see Harrison, 1969), if migration from different regions towards

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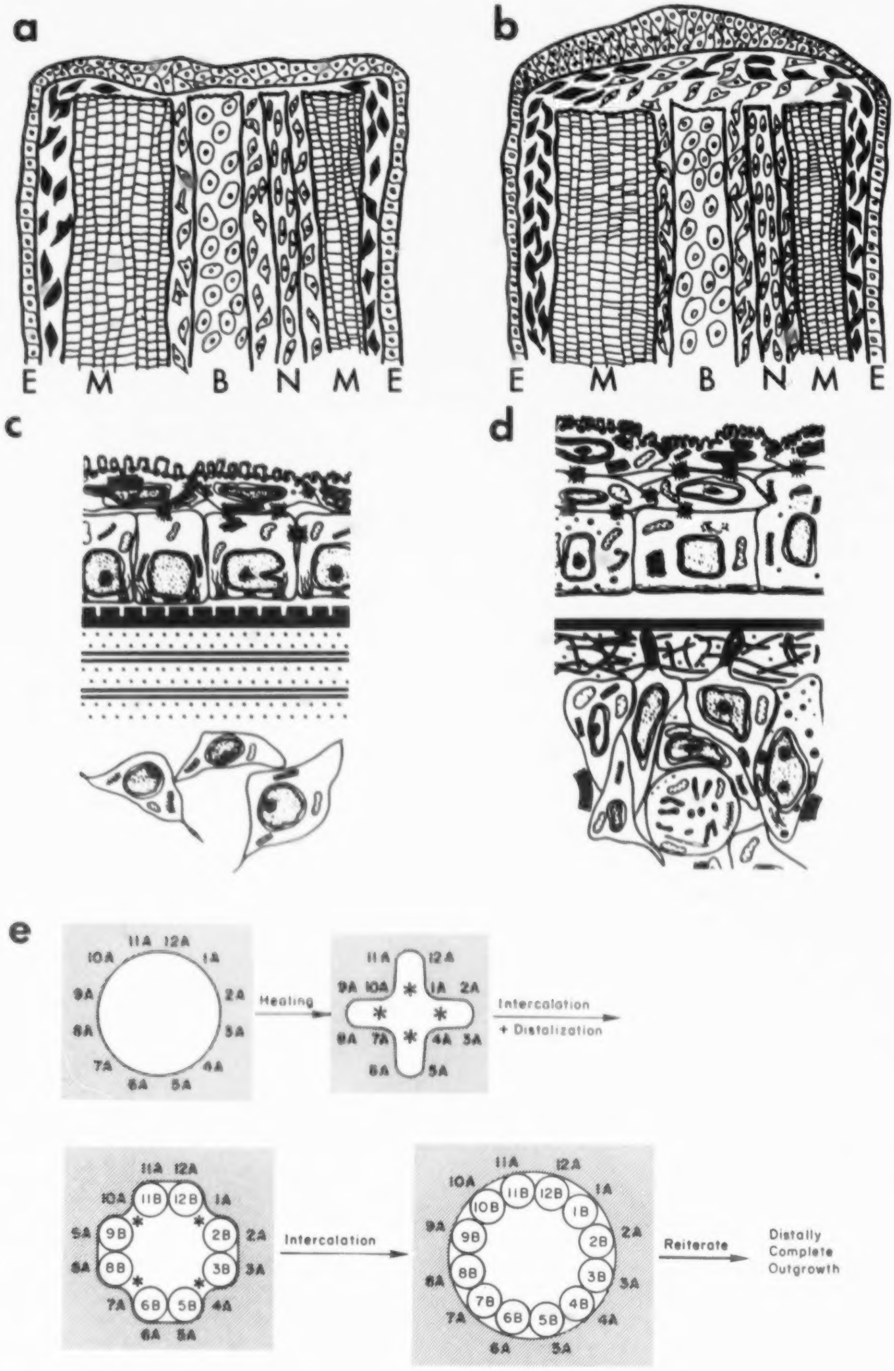
FIG. 6. A view of the initiation of outgrowth and subsequent distalization during limb regeneration and development. (a) and (b) Initiation of limb regeneration in the axolotl. Dermal cells are black. In (a) all the tissues of the limb have been transected and the amputation surface has been healed over by the wound epithelium. Several days later (b) peripheral dermal cells begin migrating centrally in the space between the wound epithelium and stump, thereby creating positional disparities and stimulating intercalation. (B, bone; M, muscle; N, nerve; E, epidermis) (c) and (d) Initiation of limb development in *Xenopus*. Prior to the appearance of the limb bud (c), epithelial cells (above) and widely dispersed mesenchymal cells (below) are separated by a complex arrangement of extracellular matrix materials. Coincident with the onset of limb bud formation (d) there is a progressive disorganization of the extracellular matrix between epithelial and mesenchymal cells in the region of the limb bud. Mesenchymal cells migrate into and accumulate in this region, and hence have the opportunity to create the positional disparities proposed to be essential to outgrowth. (From Kelley and Bluemink, 1974.) (e) The polar coordinate model for distal outgrowth. It is proposed that cells from different circumferential positions come into contact (*) and intercalate. For regenerating limbs, the migration of peripherally located cells towards the center of the amputation surface (see Fig. 5) could bring about such positional disparities. For developing limbs, the repositioning of cells beneath the epidermis at the site of future limb outgrowth (see (c) and (d) above) could bring about positional disparities. Intercalation will generate cells with new circumferential positional values which adopt more distal positional values than the pre-existing cells (the new cells adopt positional value B in this example). Since the set of circumferential positional values is asymmetrical, cells at the tip of the growing limb bud or blastema will continue to experience positional disparities, and hence continue to intercalate more distal sets of circumferential positional values. How the process of distal outgrowth might terminate is illustrated in Figure 7. (From Bryant *et al.*, 1981)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+ Distalization
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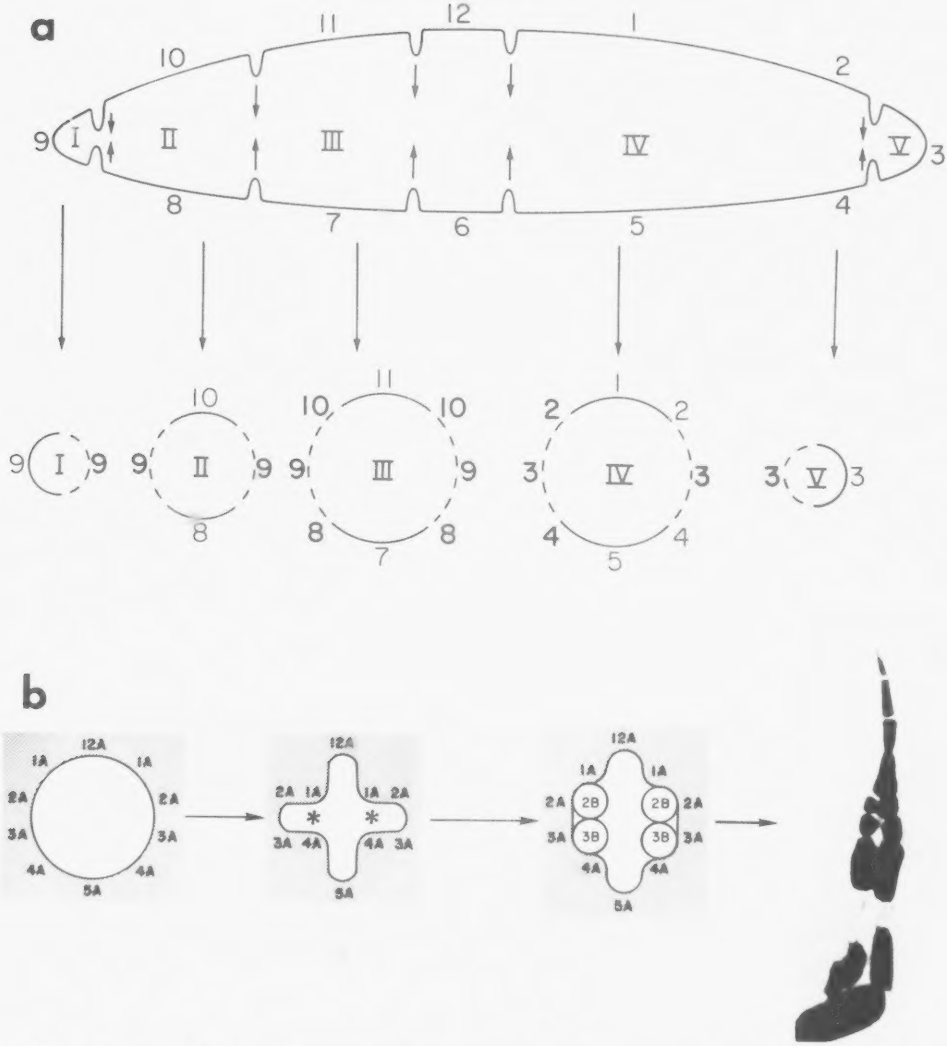

FIG. 7. Termination of distal outgrowth by digit formation. (a) Fragmentation of the limb circumference into symmetrical digit bases is proposed to occur as follows: interactions between specific circumferential positions lead to the fragmentation of the complete circle into five (or fewer, depending on the species) partial circumferences. Each isolated partial circumference will resolve positional disparities by intercalation and become symmetrical (intercalated values shown in bold face). We assume that the extreme positional values in the original limb circumference (12 and 6) do not become incorporated into any of the digit bases. In many species, cell death occurs between developing digits and this could ensure the elimination of certain positional values. Note that the digits on one side of the midline of the limb share positional values, and that peripheral digits contain fewer positional values than more central digits. (From Stock and Bryant, 1981) (b) The polar coordinate model for distal outgrowth from a symmetrical base. When the starting configuration of positional values is symmetrical, as in surgically constructed double-half limbs or as proposed for digit bases (see (a) above), then intercalation will be stimulated as described for the asymmetrical circumference in Figure 6. However, as shown here, all positional disparities will eventually be resolved and outgrowth will cease. The amount of growth which will occur prior to termination will be proportional to the starting number of different positional values, assuming that extreme values come into contact with one another. Hence, digit bases (or other symmetrical structures) with fewer positional values will terminate growth after laying down

the prospective site of limb bud outgrowth occurred at different times in development (anterior-posterior prior to dorsal-ventral). Migratory events have in fact been demonstrated to take place during the development of the eye field, thereby accounting for the apparent sequential establishment of anterior-posterior and dorsal-ventral axes in the eye (Holt, 1980).

Once limb outgrowth has been initiated by positional disparities in the two situations described above, intercalation leads to the generation of additional sets of circumferential positional values (Fig. 6e). Since the limb pattern differentiates in a proximal to distal sequence, and since termination of outgrowth by removal of the apical epidermis results in the deletion of distal structures, we assume for convenience that the new pattern is laid down in a proximal to distal sequence. Hence, reiteration of intercalation between opposed circumferential positional values at the distal tip of the mesoderm leads to the generation of successive levels of the pattern along the proximal distal axis, if each new circumferential set adopts a positional value which is more distal than the previous one (Bryant *et al.*, 1981).

Obviously, the process of distal outgrowth would keep going indefinitely if nothing intervened to halt it. It has been suggested that the way the process is halted is by the fragmentation of the circumferential sequence of positional values at the level of the digit bases (Fig. 7; Stock and Bryant, 1981). The subsets of positional values created will become symmetrical by intercalation. Further intercalation within each of the symmetrical digit circumferences will automatically lead to termination of outgrowth when all positional disparities are eventually eliminated. Direct experiments with symmetrical limbs, *i.e.*, limbs in which a partial set of the circumferential positional values is represented twice in mirror symmetry, indicate that this mechanism for growth termination is feasible, since such limbs usually show either no outgrowth, or tapering outgrowth and premature termination of the limb pattern (see Bryant *et al.*, 1982). Indirect evidence for this mechanism of digit formation can be drawn from the diverse array of different limb morphologies among the vertebrates, the vast majority of which conform to specific predictions which arise from the PCM (Stock and Bryant, 1981). These predictions are:

1. That since digits contain bilaterally symmetrical sets of positional values, they will be bilaterally symmetrical in structure. Vertebrate digits are in fact symmetrical around the dorsal-ventral axis.
2. That since peripheral digits will contain less positional information than more central digits, they will be shorter and/or less complex than the more central digits. This is the pattern that is found among present day and fossil vertebrate limbs.
3. That since some adjacent digits will have positional values in common, they will share structural similarities. Many vertebrate limbs conform to this prediction.

Finally, and perhaps most convincing, is the fact that the level of the digit bases is unique along the proximal-distal axis of the limb. It is the only known level of the urodele limb at which simple amputation does not result in the perfect regeneration of

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less proximal-distal pattern than those with a larger number of positional values. Thus, in the case of digits (see (a) above), peripheral digits will be shorter and/or less complex (*i.e.*, fewer phalanges) than their more central neighbors. At right, a regenerate from a surgically constructed double-half lower arm in the axolotl which, in accordance with the view presented above, is tapering and symmetrical. (From Bryant *et al.*, 1981) the deleted parts; amputation at this level results in regenerates possessing a variety of abnormalities including additional digits, branched or fused digits and increases or decreases in the normal number of phalangeal elements. These results suggest that wound healing at the level of the digit bases disturbs the pattern of cellular interactions that is necessary for fragmentation of the limb circumference and symmetrization of the digit bases, leading to the abnormal numbers of digits and phalangeal elements observed (Stock and Bryant, 1981).

INTRINSIC AND EXTRINSIC CONTROLS OF LIMB OUTGROWTH

The view of limb outgrowth discussed above emphasizes intrinsic mechanisms of growth control that depend upon position-specific cellular interactions between neighboring cells. As mentioned earlier, the importance of intrinsic growth control (intercalation) is not limited to the vertebrate limb. The PCM was developed to account for the regeneration of urodele limbs, cockroach legs and *Drosophila* imaginal discs. Bryant and Simpson (1984) have reviewed the roles of both intrinsic and extrinsic controls of growth during development and regeneration in a variety of other organ systems. They summarize evidence that intrinsic growth mechanisms can account not only for the initiation of growth, but also for the growth rate and final-size of structures. Examples of such phenomena come from grafting experiments between species in which grafted structures exhibit the characteristic growth parameters of the donor, rather than the host, species. Nevertheless, it is also clear that there exist a multitude of extrinsic factors that influence the ability of cells to activate these intrinsic mechanisms. Thus both intrinsic and extrinsic factors control the growth of organs and it is important to consider the various extrinsic factors known to influence limb outgrowth during development and regeneration (see Sicard, 1985).

The apical epidermis meets the criteria for an extrinsic factor without which the intrinsic programs cannot be executed. The precise role of the epidermis has not been elucidated, but it has been suggested that it maintains cells in an undifferentiated state and in the cell cycle (Tassava and Mescher, 1975; Globus *et al.*, 1980). One suggestion as to how the epidermis accomplishes this is that it produces a factor that diffuses into the subjacent, pattern forming tip of the mesoderm (see Globus *et al.*, 1980). Another is that it plays a more mechanical role by creating space at the limb tip into which apical mesoderm cells can migrate and divide (Summerbell and Wolpert, 1972). Other extrinsic factors have been identified in limb regeneration. The best studied of these is the nervous system. Singer and his colleagues have shown that a threshold quantity of innervation is required for regeneration to be initiated and to proceed through blastema formation (see Tassava and Olson, 1985). Here too the precise way in which the nerves bring about their influence is not known, although it has been suggested that they directly affect the ability of cells to go through the cell cycle. Extracts from nervous tissue have been shown to have effects on blastema cell synthetic activities (see Carlone and Mescher, 1985; Brockes and Kintner, 1986). In a recent paper, differences in the dynamics of the extracellular matrix immediately after amputation were described between innervated and denervated limbs (Mescher and Munaim, 1986), raising the possibility of a more indirect influence of the nerves via the extracellular matrix. Finally, various hormones have been described as being an essential component of the milieu in which limb outgrowth takes place (see Liversage *et al.*, 1985), but whether hormones have a direct or indirect effect on regeneration requires clarification (*e.g.*, Tassava, 1983). In no case have any of the extrinsic factors described above been shown to have any modulating effect on pattern formation itself—they either permit it or inhibit it, but never alter it in any way.

To date, vitamin A is the only extrinsic factor that has been shown to directly influence pattern formation in limbs. The role of vitamin A in normal outgrowth has not been determined, but in experimental situations its effects are dramatic. In developing limbs that are unwounded, vitamin A, in common with numerous other chemical agents, produces deficiencies in the limb pattern (Kochhar, 1977; Scadding and Maden, 1986*a*, *b*). However, if a slit is made in the anterior of the chick limb bud, and a piece of vitamin A impregnated paper is inserted, supernumerary digits are formed in addition to the normal number of digits. In fact, the application of vitamin A to chick limbs mimics the transplantation of posterior limb bud tissue to an anterior site (Tickle *et al.*, 1982, 1985; Summerbell, 1983; Eichele *et al.*, 1985). In regenerating limbs of amphibians, exposure to vitamin A during wound healing and the period of initiation of the blastema causes the formation of supernumerary structures along the proximal-distal limb axis (Niazi and Saxena, 1978; Maden, 1982, 1983). In addition, in axolotls, vitamin A stimulates the formation of posterior structures in surgically constructed limbs which, without vitamin A, would only form anterior structures (Stocum and Thoms, 1984; Kim and Stocum, 1986). One way of thinking about these results is that at a wound area anterior cells change their positional values to become more posterior, and that distal cells change positional values to become more proximal. These cells with new positional values would then interact with their neighbors and form new structures via intercalation. Thus, vitamin A as an extrinsic factor would act by altering the intrinsic mechanisms of growth control. Should this prove to be the case, vitamin A will be a valuable tool in furthering our understanding of positional information in limbs. Certainly, if we could learn how it changes positional information, we would have a more direct understanding about what positional information is. This may, however, be a long process, because from studies of the effects of vitamin A in other systems, we know that it has a multitude of diverse effects upon cellular behaviors and properties, such as altered cell motility (Thorogood *et al.*, 1982), inhibition of cell division, altered gap junctional permeability, altered cell surface glycoproteins (see Maden, 1982), and altered gene expression (see Eichele *et al.*, 1985; Maden and Summerbell, 1986). It initially will be important to understand which of these possible changes are crucial to the dramatic patterning effects of vitamin A in limbs.

Although it is our view that positional information is an intrinsic property of cells, there are other views of limb development that emphasize extrinsic factors as being the source of information. The most notable of such views is the zone of polarizing activity (ZPA) model, based on the idea that there is a diffusible chemical signal, referred to as a morphogen, that specifies positional information along the anterior-posterior axis (Tickle *et al.*, 1975). One chemical that is considered a possible candidate for this morphogen is vitamin A (*e.g.*, Tickle *et al.*, 1985), which, as discussed above, does have dramatic effects on limb pattern. Although this model can provide a reasonable explanation for the experimental data obtained from studies of the developing chick limb, it is unable to account for much of the data on developing and regenerating limbs of amphibians (see Bryant and Muneoka, 1986). On the other hand intercalation, which allows for an internally consistent explanation of limb outgrowth in amphibians, has also been utilized successfully to account for pattern formation in the developing chick limb (Iten, 1982; Javois, 1984; Javois and Iten, 1986).

SIGNIFICANCE

Apart from the inherent interest in developing an understanding of a complex, developmental event such as limb outgrowth, there are also potential practical applications for this knowledge. Urodele amphibians are the only group of vertebrates that can regenerate limbs as adults, yet we know that in this group limb outgrowth involves the same intrinsic mechanisms both for initial development of the limb and for its regeneration (Muneoka and Bryant, 1982, 1984*c*). We also can infer that limb development among the vertebrates will share common mechanisms. This inference is derived partly from the homology of the limbs of vertebrates and their relatively conserved basic structure. It also is derived from the basic pattern of development, which is similar in the cases that have been well studied. In addition, all developing limbs share similar responses to experimental manipulations, at least at some stages of their development. For example urodeles, frogs, and chicks can form supernumerary limbs in response to positional value confrontations, and they can regulate for deletions along the proximal-distal axis. Perhaps most interesting of all, they can also regenerate after amputation of the distal mesoderm if they are provided with a permissive ectodermal covering (urodeles, see Maden and Goodwin, 1980; frogs, see Maden, 1981; chicks, see Rubin and Saunders, 1972).

Although it previously has not been possible to investigate such regulative behaviors in mammalian limbs, there are indications that they are comparable to what we have discovered in amphibians and chicks. Amputation of rat or mouse limb buds of embryos cultured *in vitro* leads in some cases to the reformation of a normal-looking apical ridge, and a normal-looking limb bud (Deuchar, 1976; Burton and Bryant, unpublished). So far, it has not been possible to maintain such limbs through the period of further development necessary to assess their final pattern. However, based on experiments on the chick limb bud, it seems likely that if the apical ectodermal ridge is regenerated, then the limb mesoderm will also be able to regenerate the removed structures. Recently, using techniques for *in situ* surgery of the mouse embryo (Muneoka *et al.*, 1986*c*), we have shown that normal digits can form when most of the digit rudiment is removed, and that extra digits develop when tissues are transplanted from anterior to posterior and *vice versa* (Wanek, unpublished). Perhaps the most convincing evidence that vertebrate limbs share similar basic mechanisms for outgrowth comes from studies in which posterior tissue from a variety of vertebrate limb buds was transplanted into the anterior of a chick limb bud, causing the formation of supernumerary digits (Fallon and Crosby, 1977).

A logical extension of what we have stated thus far, is that what is true for urodeles, that limb development and regeneration use the same mechanisms, may be true for all vertebrates. If so, we next must turn our attention to why other vertebrates do not regenerate limbs, with the assumption that all the necessary genetic information for limb outgrowth is in fact present in the genome. Most studies in this area have focussed attention on the possible permissive conditions which may be inadequate for outgrowth (see Carlson, 1974*b*; Wallace, 1981). In fact, the role of the nervous system in regeneration has dominated attempts to understand regenerative failure. Hence, attempts to stimulate regeneration in non-regenerating appendages have been made by augmenting the nerve supply in one way or another. Other approaches have involved augmenting the natural injury currents present after amputation, and treating limb stumps with a variety of chemicals. Although such treatments may enhance growth, leading to the formation of some additional structure distal to the amputation plane, they have been unsuccessful in causing the development of patterned outgrowths. Perhaps the most promising reports to date using such approaches involve the use of vitamin A and DMSO in postmetamorphic frogs (Cecil and Tassava, 1986*a*, *b*). Here, structures resembling the normal limb pattern have been obtained in animals that normally would not exhibit regeneration. It is of interest that of all the exogenous factors known to affect limb outgrowth, vitamin A is the only one which has so far been identified as influencing positional information (see above). In other words, the one exogenous factor that appears to influence the intrinsic controls of limb outgrowth is also one of the two factors that have been the most successful to date in stimulating regeneration.

Several other lines of reasoning indicate that we should seek the causes and cures of regenerative failure in the intrinsic mechanisms as well as the extrinsic factors related to limb outgrowth. In *Xenopus* limbs, which regenerate well during larval stages but show progressive loss of regenerative ability, extrinsic factors seem unlikely to be limiting. For example, it has been shown that during the period of regenerative decline, limbs do not produce randomly defective regenerates (Muneoka *et al.*, 1986*b*). The regenerated limbs exhibit progressive loss of anterior structures that are normally the last to form during outgrowth. Thus it is possible that the ability to respond to positional disparities has become impaired before all remaining disparities are resolved, resulting in premature termination of growth and patterning. Further support for this idea is found in experiments in which different amounts of tissue were removed from the foot plate of larval *Xenopus*, leading to either a small or a large positional disparity (Muneoka *et al.*, 1986*b*). Despite the fact that the wound sizes were similar in these experiments, as were all extrinsic factors, limbs containing large positional disparities exhibited a regenerative response more frequently than those with small disparities. Finally, despite the temptation to attribute regenerative failure in frogs to the changing milieu at metamorphosis, it has been shown that repeated amputation of limbs during the period of metamorphosis allows limbs to keep on regenerating well past the time at which the response would normally have terminated (Kollros, 1984). Perhaps most definitively, limb buds transplanted to postmetamorphic limb stumps, and then amputated, undergo regeneration as if they had remained on the larva (Sessions and Bryant, unpublished), showing that all of the extrinsic conditions in the postmetamorphic animal are fully permissive for limb outgrowth.

In summary, we believe that progress towards the goal of stimulating regenerative limb outgrowth in non-regenerating vertebrates, including mammals, will be contingent upon a better understanding of the intrinsic mechanisms involved in outgrowth, and in the changes that occur in these processes as regenerative ability is lost.

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Developmental Biology and Human Concerns[1]

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SYNOPSIS. There is a large natural loss of human embryos in early gestation. Most conceptual losses occur before pregnancy has been diagnosed in the woman. It is now acknowledged that chromosomal aberrations are the major etiologic agents responsible for spontaneous abortions. Fully 50 percent of naturally aborted embryos in the first trimester have an abnormal karyotype. Most of the chromosomal errors that have been identified in abortuses are only rarely seen in livebirths. Natural *in utero* selection is relentless in eliminating 99 percent of the chromosomally abnormal conceptuses through spontaneous abortion. The birth of affected offspring that escape nature's screening mechanism can be averted by the option of prenatal diagnosis. The thrust of prenatal diagnosis is to prevent the tragic impact of debilitating genetic disorders. But not all at-risk parents wish to avail themselves of prenatal diagnosis because they are unwilling to accept the choice of therapeutic abortion. Prevention of a genetic disorder before implantation would obviate the necessity of an abortion at a later stage of pregnancy. With this perspective, the correction of the basic genetic flaw by replacing the faulty gene with a functioning allele is an attractive alternative. Notwithstanding the imprecise technology that presently serves to caution against immediate application, gene therapy is a reasonable and natural extension of efforts to ameliorate the effects of severe inherited disorders.

INTRODUCTION

The subject of prenatal loss of embryos, or *pregnancy wastage* as it is often termed in the medical literature, is of universal and recurrent interest. In the early years of this century, prenatal deaths were generally attributed to faulty implantation associated with some pathologic process in the uterus (Mall and Meyer, 1921). After this early phase the pendulum oscillated to the opposite extreme so that attention was focused on mishaps in the egg itself (Streeter, 1931). Investigators continued to assess and debate the importance of sundry factors in prenatal loss, but it was not until the advent of modern cytogenetics in the 1950s that it became abundantly clear that defects inherent in the embryo account for the major portion of prenatal mortality. Once considered exceptionally rare and relatively unimportant, chromosomal aberrations now loom as the most prominent cause of embryo loss. The realization of the cardinal role of chromosomal aberrations was accompanied by the perception that nature preferentially selects against abnormal embryos and fetuses. In recent years, developmental biologists have come to appreciate the screening role of natural selection. The forces of natural selection ensure that the vast majority of human conceptuses with major chromosomal anomalies do not survive to term (French and Bierman, 1962; Roberts and Lowe, 1975; Schlesselman, 1979; Chandley, 1981).

EARLY PREGNANCY LOSS

Statistical considerations

We value the meticulous work of Hertig and his colleagues (1956, 1959), who described in rich detail the structural malformations of early embryos recovered from fertile women who had undergone elective hysterectomy. At least 30 percent of the pre-implantation embryos and early implanted embryos were grossly abnormal and unlikely to have remained viable through pregnancy. In 1967, Hertig estimated that 16 percent of human oocytes fail to become fertilized, about 10 to 15 percent cleave but are unable to implant, and only 42 percent are of such viability

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[1] From the Symposium on *Science as a Way of Knowing—Developmental Biology* presented at the Annual Meeting of the American Society of Zoologists, 27–30 December 1986, at Nashville, Tennessee.

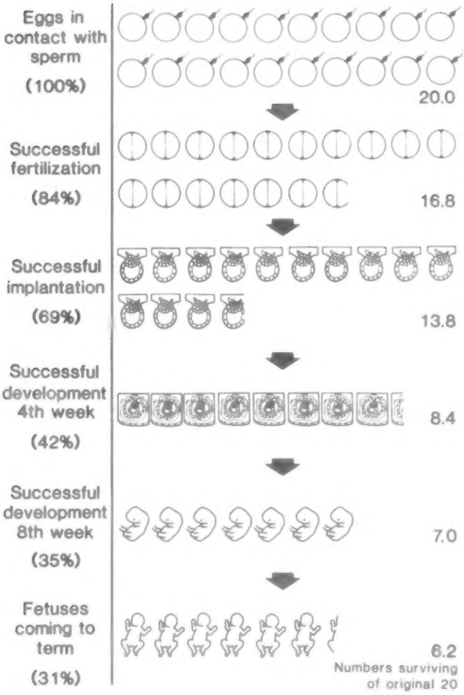

FIG. 1. The fate of 20 eggs produced by women who are reproducing naturally. Under conditions optimal for fertilization and development, only 6.2 eggs of the original 20 (31%) develop successfully to term.

TABLE 1. *Table of intrauterine deaths per 100 ova exposed to the risk of fertilization.*

Weeks after ovulation	Survivors[a]	Failures[b]
	100	16[c]
0	84[d]	15
1	69[e]	27
2[f]	42	5.0
6	37	2.9
10	34.1	1.7
14	32.4	0.5
18	31.9	0.3
22	31.6	0.1
26	31.5	0.1
30	31.4	0.1
34	31.3	0.1
38	31.2	0.2
Livebirths	31	

Data from Leridon (1977).
[a] Pregnancies still in progress.
[b] Spontaneous abortions.
[c] Not fertilized.
[d] Number fertilized.
[e] Number implanted.
[f] Expected times of menses.

as to cause the woman to miss her expected menstrual period. Hertig's startling conclusion was that most conceptual losses occur subclinically—that is, before pregnancy has been diagnosed in the woman.

The data are as yet too imprecise to provide a true rate of early pregnancy loss in the human female. The available information, however, does permit a mathematical estimation that appears to reliably approximate natural events. Confidence has been placed in Leridon's table of intrauterine death (1977). As seen in Table 1, Leridon explores what happens to 100 eggs produced by women who are reproducing naturally. His initial premise, following Hertig, is that 16 of the 100 eggs will fail to be fertilized, even under optimal conditions. Of the 84 eggs that are fertilized, 15 will fail to implant. Of the 69 embryos implanted at the end of the first week, 27 of these characteristically will find the lining inhospitable. Thus, up to the second week after ovulation, only 42 eggs of the original 100 will still be viable. Stated another way, by the time pregnancy is recognizable, more than half the eggs have been lost. At eight weeks' gestation when the embryo is now termed a fetus, about 65 eggs of the original 100 will have failed to survive. Fortunately, the incidence of spontaneous abortion during the fetal period is very low. At the end of gestation, the probability of a livebirth is only 0.31, or 31 percent. For ease of comprehension, the numerical information in Table 1 is pictorially represented in Figure 1, in which the baseline is an original 20 eggs rather than 100 eggs.

There is now experimental evidence to support the foregoing statistical model of pregnancy loss. Measurements of human chorionic gonadotropin (hCG) have been used to assess early embryo loss. In humans, pregnancy is acknowledged biochemically by the trophoblastic production of hCG at an early stage of pregnancy. Accordingly,

TABLE 2. *Frequencies of different types of chromosomal abnormalities in 463 human spontaneous abortions with abnormal karyotypes.*

Abnormality	Chromosome number	Percentage of total abnormalities
Monosomy	45	24.2
Trisomy	47	44.5
Double trisomy	48	1.9
Triploidy	69	15.1
Tetraploidy	92	7.1
Mosaicism	—	2.6
Structural aberrations	46	4.3

Data from Hassold *et al.* (1980*a*).

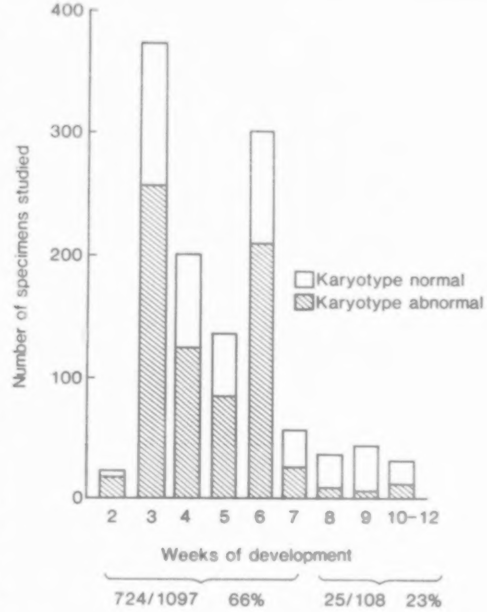

FIG. 2. Frequency of chromosomal abnormalities in spontaneous abortuses in relation to weeks of development. (From Boué and Boué, 1976)

urine samples can be collected throughout the luteal phase of the menstrual cycle from normal women wishing to conceive. In one such study, 50 of 152 pregnancies (33%) were early abortions diagnosed by elevated levels of hCG (Miller *et al.*, 1980). In another report, 62 percent of the conceptuses were lost prior to 12 weeks, of which the majority occurred subclinically without knowledge of the mother (Edmonds *et al.*, 1982). Not only do these studies support the mathematical estimations, but reveal as well a high rate of embryo loss in humans that far exceeds pregnancy loss in many other mammalian species (Bond and Chandley, 1983). Malformations in early human embryos may be the norm rather than the exception.

Etiology of pregnancy loss

With the rapid advances in chromosome methodology in the 1960s, it was inescapable that the human chromosome complement would be examined in the cells of spontaneous abortuses. To most clinicians the results of chromosome analysis were astonishing. Several thorough investigations have revealed that 50–70 percent of first-trimester spontaneous abortuses are chromosomally abnormal (Carr, 1967; Kajii *et al.*, 1973; Therkelsen *et al.*, 1973; Boué and Boué, 1976; Hassold *et al.*, 1980*a*; Stein, 1981). The data demonstrate convincingly that chromosomal aberrations are the major etiologic agents responsible for naturally occurring abortions. We may cite the extensive findings of Boué and Boué (1976) on the incidence of chromosomal anomalies in human abortuses. Among 1,097 specimens between the second and seventh weeks of gestational age, 724, or 66 percent, had an abnormal karyotype (Fig. 2). The incidence of chromosomal abnormalities fell to 23 percent among the 108 abortuses between 8 and 12 weeks of age. Most types of chromosomal abnormalities are incompatible with survival to an advanced stage of pregnancy.

Reports on the variety and distribution of chromosomal errors are remarkably consistent (Bond and Chandley, 1983; Hassold and Jacobs, 1984; Boué *et al.*, 1985). In virtually all studies, more than 40 percent of the spontaneous abortuses have a trisomic constitution ($2n + 1$), possessing 47 chromosomes instead of the normal number of 46 (Table 2). The additional chromosome usually arises as a consequence of meiotic nondisjunction, an event that apparently increases with advancing maternal age. The underlying cause of the dramatic increase in trisomic conceptions among older women remains one of the more important unanswered questions.

The frequencies of trisomies in different autosomal groups vary widely: trisomies for chromosomes 13, 16, 18, 21, and 22 occur most often, especially chromosome 16. For reasons that are not understood, chromosome 16 appears to be particularly vulnerable to nondisjunction; trisomy 16 accounts for one-third of all autosomal trisomic abortuses. Interestingly, trisomy 16 among abortuses shows little association with increasing maternal age, which suggests that an unusual age-independent mechanism is responsible for this extraordinarily common trisomic condition (Hassold *et al.*, 1980*b*). There is no autosomal trisomic condition in humans that is not associated with marked developmental disturbances. The three autosomal trisomies that occasionally survive to term are 13, 18, and 21, and these are sufficiently common at birth to have well-described syndromes (Patau, Edwards, and Down, respectively).

Autosomal monosomies ($2n - 1$) are rarely recorded; the vast majority perish very early before pregnancy is clinically recognizable. Whereas autosomal monosomies are scarcely encountered, monosomy X (or 45,X) accounts for 20–25 percent of chromosomally abnormal abortuses (Table 2). Monosomy X arises far more often than is predictable on the basis of an exclusively nondisjunction origin. Although this sex-chromosome monosomy is largely incompatible with life *in utero*, those 45,X females viable at birth (manifesting Turner syndrome) have inexplicably relatively few life-threatening anomalies. Among the more common sex-chromosome aneuploids, those that can survive to term are the 45,X and 47,XXX conditions in females and the 47,XXY and 47,XYY conditions in males. Among liveborn females, monosomy X (45,X) occurs with a frequency of one in 6,000, which is considerably rarer than the 47,XXX constitution, which arises once in 1,000 livebirths. The great loss of 45,X fetuses during the prenatal period accounts for the relatively low incidence of such monosomic females among the newborn.

Polyploidy represents another major category, with triploid and tetraploid karyotypes occurring in about 15 and 7 percent, respectively, of all clinically recognized abortuses (Table 2). Triploid fetuses seldom survive the third trimester of pregnancy, while tetraploid conceptuses are lethal at an early gestational stage. Structural chromosomal rearrangements, such as translocations and inversions, are less common in abortuses than numerical abnormalities. They are, however, of clinical importance since they can be perpetuated from one generation to the next.

Most of the chromosome abnormalities that have been identified in abortuses are only rarely seen in livebirths. We may estimate conservatively that one in five of all conceptuses carries a chromosome abnormality. At birth, the incidence declines dramatically to about one in 200, most with significant physical impairment and mental incapacitation.

Natural selection

It may be disconcerting to learn that there is a large natural loss of embryos in pregnancy. The important point, however, is the high efficiency with which nature eliminates abnormal embryos during the course of pregnancy. For every 1,000 chromosomal abnormalities that are present in embryos in the uterus, only six are expected to survive to the point of a livebirth. Thus, 99.4 percent of the chromosomal abnormalities are eliminated naturally through spontaneous abortion (Lubs, 1977).

We may now direct our attention to the 0.6 percent of newborn infants affected with a chromosomal abnormality. As seen in Figure 3, some members of this affected

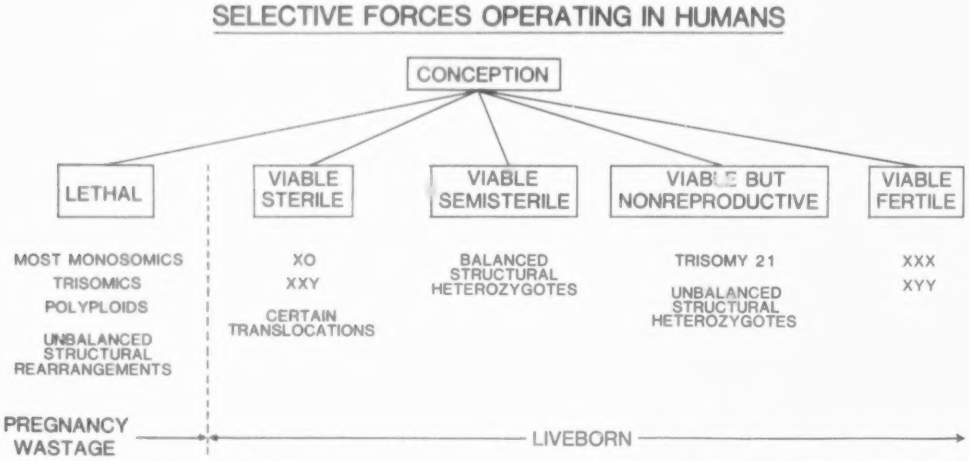
SELECTIVE FORCES OPERATING IN HUMANS
CONCEPTION
LETHAL
VIABLE STERILE
VIABLE SEMISTERILE
VIABLE BUT NONREPRODUCTIVE
VIABLE FERTILE
MOST MONOSOMICS
TRISOMICS
POLYPLOIDS
UNBALANCED STRUCTURAL REARRANGEMENTS
XO
XXY
CERTAIN TRANSLOCATIONS
BALANCED STRUCTURAL HETEROZYGOTES
TRISOMY 21
UNBALANCED STRUCTURAL HETEROZYGOTES
XXX
XYY
PREGNANCY WASTAGE
LIVEBORN

FIG. 3. Natural selection operates in human populations to ensure the reduced survival and minimal reproductive potential of individuals with major chromosomal abnormalities. (After Chandley, 1981)

group comprise the 45,X (Turner) and 47,XXY (Klinefelter) conditions. These individuals are effectively eliminated from the reproductive pool by their sterility. In contrast, semi-sterile carriers of balanced translocations tend to be normal phenotypically, but are at risk of having abnormal children. The risk, however, is reduced appreciably by the segregation of unbalanced chromosome complements in gametes that are likely to be inviable.

From the perspective of natural selection, the XXX females and the XYY males represent an instructive group. Both types of individuals are usually fertile, and their children are chromosomally normal (XX daughters and XY sons). Some mechanism operates during meiosis to eliminate the extra X chromosome from the egg, or the extra Y chromosome from the sperm. Thus, natural selection has modified the meiotic divisions to ensure that only normal haploid gametes are produced by XXX females and XYY males.

The salient feature of the foregoing considerations is the efficacy with which natural selection eliminates chromosomally abnormal conceptuses—largely through pregnancy wastage and augmented by sterility or a special form of meiosis that eliminates disomic chromosome complements. Nature has created a great barrier to the perpetuation of chromosomally abnormal offspring. Natural selection, however, is not perfect. Some chromosomally abnormal fetuses escape nature's screening mechanism and survive to term. Down syndrome represents one of nature's failures; only 80 percent of Down infants are aborted spontaneously. About 20 percent go on to be liveborn. The urgent question is whether we should assume the responsibility for ameliorating nature's shortcomings by further limiting the opportunities for the survival of major chromosomal anomalies. An affirmative answer was given to this question when prenatal diagnosis of genetic disorders entered the mainstream of medical practice in the 1970s.

PRENATAL DIAGNOSIS

Among the dramatic achievements in the last two decades has been the discovery of precise and relatively safe ways of diagnosing genetic disorders *in utero*. From a modest beginning in which a few chromosomal aberrations and a limited number of enzyme deficiencies were analyzed, the

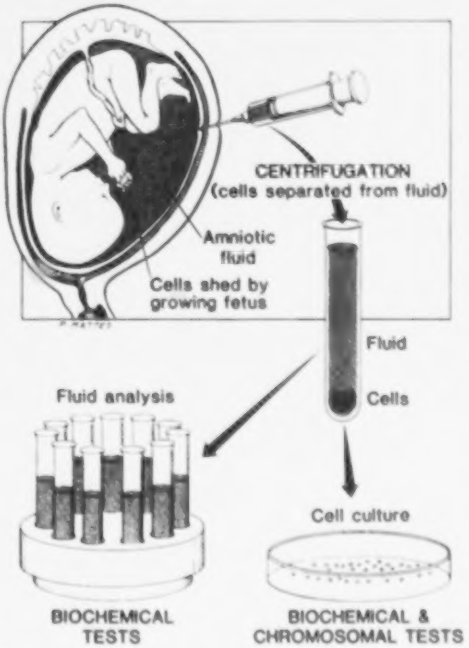
CENTRIFUGATION
(cells separated from fluid)
Amniotic fluid
Cells shed by growing fetus
Fluid
Cells
Fluid analysis
Cell culture
BIOCHEMICAL TESTS
BIOCHEMICAL & CHROMOSOMAL TESTS

FIG. 4. Technique of amniocentesis. A sample of amniotic fluid is withdrawn by inserting a needle through the abdomen into the uterine cavity at 14–16 weeks of pregnancy. The procedure is precarious earlier than 14 weeks' gestation because of the small amount of amniotic fluid in relation to the size of the fetus.

scope of prenatal diagnosis has expanded to include the important hemoglobinopathies and varied structural deformities, prominent among them the neural tube defects (Simpson and Verp, 1982; Epstein *et al.*, 1983; Wald, 1984). Accurate diagnoses have been achieved through *amniocentesis*, a procedure involving the removal of fluid from the amniotic cavity surrounding the developing fetus. Cells are continually shed into the amniotic fluid and these free-floating cells have been ascertained to be of fetal origin (Fig. 4).

The amniotic fluid cells slough off primarily from the fetal skin and amniotic sac; some are derived from the intestinal and urinary tracts of the fetus. Although the amniotic fluid exists from an early stage in pregnancy, the quantities are at first very small and difficult to obtain without appreciable risk to the fetus. At about 10 weeks the volume of amniotic fluid is only about 30 ml. It is customary for obstetricians to wait until the 14th or 15th week of pregnancy when the volume of amniotic fluid has increased substantially. At the 14th week, the fluid attains a volume of 175–225 ml (6–8 oz), from which as much as 25 ml can be safely removed. In laboratory testing, the fluid is studied for elevated levels of alpha-fetoprotein (associated with neural tube defects) and the cells are cultured for chromosomal and enzymatic analyses. Approximately 24 days are required to complete the chromosomal studies and 35 days for the biochemical assays. Since the culturing of cells is time-consuming, enzyme analyses are performed, where feasible, on the uncultured fetal cells of the original sample.

As listed in Table 3, there are several compelling situations that warrant prenatal diagnosis. A clear-cut indication for amniocentesis is the increased risk in older women of bearing offspring with chromosomal aberrations, particularly trisomies. In many clinics, amniocentesis is routinely recommended to women over 35 years of age. The increased risk associated with advanced maternal age has been documented for autosomal trisomies 13, 18, and 21, and for the sex chromosome imbalances 47,XXX and 47,XXY (Schreinemachers *et al.*, 1982).

If the cultured amniotic cells reveal a serious chromosomal or biochemical abnormality, the prospective parents can explore at least two options: they can prepare themselves for the special concerns in caring for a child with a birth defect, or they can terminate the pregnancy. When prenatal diagnosis forewarns of a severe handicap, the choice has usually been for a therapeutic abortion. Nonetheless, it is important to support, with all available information, the mother who chooses to continue a pregnancy in which the fetus is demonstrably affected. Fortunately, the vast majority of monitored pregnancies are uneventful, and the high-risk parents are relieved of the anxiety caused by the threat of a serious disorder in their child. In this respect, prenatal diagnosis may be viewed as birth-facilitating rather than birth-preventing. Stated another way, the availability of prenatal diagnosis, particularly for the older woman, makes conception acceptable where previously it was often avoided because of the risk, even when small, of bearing an affected child.

The findings of several studies indicate that amniocentesis is a safe and highly accurate procedure that does not significantly increase the risk of fetal injury or loss. Given the high rate of naturally occurring abortion, it is difficult to demonstrate unequivocally that a spontaneous abortion after amniocentesis is a direct consequence of the procedure. The question that can be answered is whether fetal loss occurs more often after amniocentesis than usual. Statistically, there is no demonstrable increased risk of abortion following amniocentesis (NICHD National Registry, 1976). In a large sample in the United States, 3.5 percent of pregnant women who underwent amniocentesis experienced fetal loss subsequent to the procedure. But the frequency in control subjects is 3.2 percent; the difference between 3.2 and 3.5 is not statistically significant.

The paramount concerns of amniocentesis are twofold: the procedure is performed in the second trimester (14–16 weeks' gestation), and the anxious parents typically wait two to three weeks to obtain a report. It would be clearly desirable to perform prenatal diagnosis before the 12th week of gestation, so that mothers could opt for first-trimester termination of pregnancy, a less psychologically stressful undertaking for the mother than the midtrimester procedure (Jones *et al.*, 1984). Many mothers feel that a first-trimester abortion of an embryo is morally more acceptable than the termination of the life of a fetus in midgestation. Recently, in several obstetric centers, there has been renewed interest in *chorionic villus sampling*, a technique which, in former years, was associated with a relatively high rate of complications. In this procedure, a sample of fetal tissue is obtained, between the 9th and 11th weeks of gestation, from the developing chorionic villi by passing a soft, flexible catheter (with ultrasound guidance) through the cervix into the placenta (Fig. 5). Aspiration usually yields 10 to 25 mg of chorionic tissue, which is sufficient for *direct* chromosomal, biochemical, and DNA (restriction enzyme) studies (Kazy *et al.*, 1982; Jackson, 1985). In the absence of the necessity for tissue culture, a diagnosis can be rendered within 48 hr after sampling (Simoni *et al.*, 1983). Although the risk to the fetus of chorionic villus sampling has been reported to be greater than that of amniocentesis, the safety of the procedure continues to be the subject of critical evaluation (Modell, 1985).

TABLE 3. *Indications for midsemester amniocentesis.*

—Maternal age over 35 years
—Previous birth of a trisomic child or other chromosomal abnormality
—Carrier parent with a balanced chromosomal translocation
—Raised maternal serum alpha-fetoprotein
—Previous child with a neural tube defect
—Previous child with a metabolic disorder than can be diagnosed *in utero*
—Woman who is a carrier for an X-linked recessive disorder (such as Duchenne muscular dystrophy)
—Habitual aborters (three or more spontaneous abortions previously)
—Multiple congenital anomalies in a previous child

The termination of pregnancy on the basis of the results of amniocentesis or chorionic villus sampling has raised ethical issues. Some of the moral concerns relate to abortion in general, whereas others are unique to the prenatal diagnosis of an abnormal fetus. The latter issue relates to the kinds and degree of intellectual and

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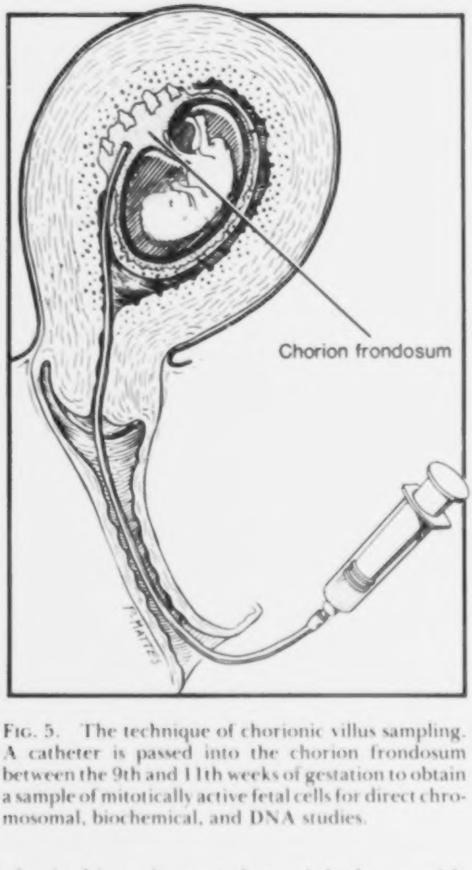

FIG. 5. The technique of chorionic villus sampling. A catheter is passed into the chorion frondosum between the 9th and 11th weeks of gestation to obtain a sample of mitotically active fetal cells for direct chromosomal, biochemical, and DNA studies.

physical impairment that might be considered adequate for therapeutic abortion. Some conditions, such as Tay-Sachs disease, are profoundly incompatible with a meaningful existence. Other conditions, such as Down syndrome, do not exclude all capacity for normal human satisfactions. Who decides when life is not worth saving? Broad support may be marshalled for the view that the decision-making process is highly personal, revolving around the emotional resources of the parents faced with the possible lifetime responsibilities of a child with a severe defect (Volpe, 1984). The parents are the ones who must live with, and are most affected by, their decision.

TABLE 4. *Risk of having a liveborn child with Down's syndrome and other major chromosomal abnormalities by 5-year maternal age intervals.*

Maternal age	Risk of Down's syndrome	Total risk for all major chromosomal abnormalities*
20	1/1,923	1/526
25	1/1,205	1/476
30	1/885	1/384
35	1/365	1/178
40	1/109	1/63
45	1/32	1/18

Data from Hook and Chambers (1977) and Hook (1981).

* Chromosomal abnormalities other than Down's syndrome include trisomy 13 (Patau syndrome), trisomy 18 (Edwards syndrome), and 47,XXY.

We may consider some of the sensitive issues raised by prenatal diagnosis by inquiring into selected genetic disorders—namely, Down syndrome, cystic fibrosis, and Huntington's disease.

Intrauterine diagnosis of Down syndrome

Down syndrome is the most common multiple congenital disorder associated with severe mental retardation (Volpe, 1986). The disorder occurs when chromosome 21 (or a segment of its long arm) is present in three copies (trisomy 21). It is the condition for which amniocentesis is most commonly undertaken. In the United States, the overall incidence of Down syndrome is about one in 700 livebirths. The frequency of Down syndrome increases markedly with the age of the mother, occurring in about one in 1,900 births at maternal age 20, to one in 100 births at age 40. Among 45-year-old women, one in 30 infants may be expected to be affected with this syndrome (Table 4).

Since 1960 the mean maternal age for all livebirths has declined substantially because of the decreasing number of children born to women over 35 years of age. In the United States, the proportion of all births to women 35 and older has decreased unremittingly from 10 percent in 1960 to 4 percent in 1977 (Luthy *et al.*, 1980). Stated another way, women younger than

35 are currently responsible for 96 percent of all births. Given the lower average age of childbearing women, the expectation has been that the incidence of Down syndrome would also decrease. The anticipated decline has materialized. The percentage of Down infants born to women 35 years or older has been reduced nearly in half—from 43 percent in 1960 to 23 percent in 1977. This substantial reduction during the past two decades has occurred not only because of the changing reproductive attitudes and behaviors of couples but also as a consequence of the availability of prenatal diagnosis.

It is likely that public acceptance of prenatal diagnosis will increase in forthcoming years. As more couples elect to limit family size, a higher premium will be placed on each conception. Women who continue to seek career opportunities will doubtless delay childbearing until the latter part of their reproductive years. Indeed, an increasing number of women are postponing childbearing into the fourth decade (National Center for Health Statistics, 1982). It is this group of women who are at a relatively increased risk for bearing children with chromosomal abnormalities.

The trend in the recent past toward reductions in the incidence of Down births to women over 35 years of age is expected to reverse in the foreseeable future as the large cohort of women born during the post-World War II boom (second half of the 1940s) moves into the over-35 category. The 35–45 age category is projected to increase from its 1980 level of 19 million to about 30 million by 1995, an increase greater than 50 percent (Huether, 1983). In the absence of any reduction in Down births through prenatal diagnosis, the number of Down births is estimated to increase from about 4,300 in 1979 in the United States to about 5,300 in 1990. Only a dramatic increase in prenatal diagnosis—the utilization of amniocentesis by 75 percent of expectant mothers over 35—can offset the projected twentyfold increase in Down births resulting from the anticipated larger number of births to women over 35 years of age.

Presently in the United States, the percentage of older at-risk women who avail themselves of prenatal diagnosis varies from 6 percent to 40 percent, depending upon the region of the country. The utilization rate is very low in rural areas, as expectant mothers in nonurban regions simply do not have access to facilities in which prenatal diagnostic procedures are performed. National opinion surveys have indicated that the vast majority of women approve and support the application of amniocentesis in high-risk pregnancies. Nevertheless, in several regions in the nation, many obstetricians have not referred a single patient for amniocentesis (Adams *et al.*, 1981; Bernhardt and Bannerman, 1982).

Cystic fibrosis

An increasing number of investigators have sought to develop a test for the prenatal detection of cystic fibrosis (Brock *et al.*, 1985). This condition is one of the most common inherited disorders in Caucasian populations, affecting one of every 2,000 livebirths. As currently understood, cystic fibrosis is inherited as an autosomal recessive trait, with no suggestion of multilocus heterogeneity. Despite many investigations, there is still no clear indication of the nature of the basic biochemical defect. In the absence of information on the gene product, it is not possible to use reverse transcriptase to synthesize a complementary DNA (cDNA), from which a radiolabeled probe can be prepared to locate the mutant gene on a specific chromosome. Nevertheless, certain molecular strategies can be employed that permit localization of the gene without knowledge of what the gene does.

Chromosome assignment can be made on the basis of linkage analysis—that is, uncovering DNA markers (DNA polymorphisms) that are closely linked to the mutant gene that causes the disease. The DNA markers are inherited changes, generally single base-pair mutations, that create or abolish recognition sites for restriction endonucleases. The addition or loss of these restriction sites results in different sizes of DNA fragments, called "restriction fragment length polymorphisms," or RFLPs. The RFLPs serve to differentiate between the chromosome carrying the normal gene and the homologue bearing the defective gene. The DNA polymorphisms are themselves not of any importance in the disease process. They are inherited bystanders that serve as effective markers of the faulty gene of interest provided they occur in families at risk (Botstein *et al.*, 1980; Gusella *et al.*, 1980; Davies, 1981; Shows *et al.*, 1982).

The year 1985 witnessed the first demonstration of tight linkage between the locus for cystic fibrosis and a DNA sequence polymorphism, uncovered almost simultaneously by no less than four different research groups (Knowlton *et al.*, 1985; Tsui *et al.*, 1985; Wainwright *et al.*, 1985; White *et al.*, 1985). All four teams have placed the cystic fibrosis gene in the middle portion of the long arm of chromosome 7. The localization of the gene will facilitate the subsequent isolation of the disease gene and the identification of the primary gene product. Knowledge of the chromosome location of the mutant gene also permits the development of a diagnostic test for family members at risk.

The thrust for prenatal screening may well be a two-edged sword. The survival of patients with cystic fibrosis has improved dramatically in the last two decades. In the mid-1960s, affected children could not look forward to reaching the age of 10 years, despite the best efforts of the medical profession. Presently, with increased clinical awareness and aggressive therapeutic measures, more than half the babies born with this disorder today can expect to live beyond the age of 21 years. Cystic fibrosis has graduated from the pediatric clinic and has become a disease of adults. The disorder, however, still retains its distressing aspects, and the greater longevity may be viewed as imposing greater rather than lesser burdens on the patients and their families. Given these circumstances, what will be the attitude of parents if prenatal diagnosis becomes a reality and a cure has yet to be found?

The consensus of medical geneticists is that every effort should be made to promote continued research in prenatal diagnosis. Nearly all young mothers with a cystic fibrosis child wish to become pregnant again *without* the 1 in 4 risk of another affected child. Most mothers want more assurance than mere statistical probabilities. It is not uncommon for parents to profess that they do not wish to expose another child to the symptoms and distresses of cystic fibrosis. Many parents have been influenced by the feelings of affected older children, many of whom are desperately anxious that a younger brother or sister should not have cystic fibrosis.

Huntington's disease

Huntington's disease is a progressive neurodegenerative disorder of late onset. The symptoms usually develop in an affected person between the ages of 30 and 45. There is no cure and the progress of the disease is relentless, leading to a terminal state of helplessness. Huntington's disease is transmitted through a single dominant gene with virtually full penetrance. The completeness of penetrance means that those individuals at risk have a 50 percent probability of manifesting the disease in later life.

One of the most exciting single events in current research on Huntington's disease has been the chromosomal localization of the gene for the disorder. In 1983, studies by James Gusella and his colleagues at Massachusetts General Hospital revealed that the gene for Huntington's disease is linked to a polymorphic DNA marker associated with chromosome 4 (Gusella *et al.*, 1983). The success of the linkage analysis was in large measure due to the existence of two large multigeneration families—one in the United States and the other in Venezuela—with extended histories of Huntington's disease. The informative population in Venezuela has the largest known concentration of Huntington's disease in one family. In a kindred numbering over 3,000 since the early 1800s, there are presently 100 living descendants with Huntington's disease. An interdisciplinary group of scientists, including Nancy S. Wexler, has been involved in collecting pedigree information, tissue samples, and clinical data over a four-year period (Wexler, 1985).

The possibility of an accurate test for the presymptomatic detection of Huntington's disease has sparked debate over the ethics of using screening techniques to detect an incurable disease. Some investigators firmly maintain that a screening test should be withheld until something tangible, notably a cure, can be offered to those who show a positive response. The practical usefulness of the test cannot possibly compare with the psychological impact that the results would likely have on the asymptomatic person at a young age. If the test is negative, that person can have children and feel secure in the knowledge that the offspring also will be unaffected. If the test is positive, the person must live with the grim certainty that the only path that lies ahead in life is insidious physical and intellectual decline. The positive aspect of the test, therefore, can be worse than the current situation, which is distressful enough.

At present, in the absence of testing, all offspring of parents with Huntington's disease wait anxiously throughout their lives to learn if they have been spared. All of them, however, can have some degree of optimism that they might escape, knowing that statistics give them a 50-50 chance. The screening test will clearly establish the ones who *cannot* be optimistic about the future. Is this knowledge worse than not knowing? The incisive question is: Can an asymptomatic young person tolerate a positive response that would be provided by a reliable diagnostic test? The emotional impact of an early diagnosis may be too much to endure, particularly if an at-risk expectant mother decides to test her unborn baby for the gene. If the test is positive for the fetus, then the at-risk mother also has the fatal gene!

GENE THERAPY

Somatic cell therapy

During the past several years, few scientific subjects have attracted as much widespread attention as has the technology of recombinant DNA (Davies, 1981; Miller, 1981; Antonarakis *et al.*, 1982). When first introduced the novel procedure evoked heated debate over its possible perils, but the controversy has largely subsided. Recombinant DNA technology is currently acknowledged as one of the most powerful tools for revolutionizing the management of inherited disorders. Such management has been called *gene therapy*, a popular topic both in the scientific literature and the lay press (Motulsky, 1983; Anderson, 1984; Culliton, 1985*a*, *b*).

In theory, the principle of gene therapy is deceptively simple: a malfunctioning gene is replaced by, or compensated by, a properly functioning gene. In reality, however, the task is formidable. One of the more serious impediments is our limited understanding of how a given gene interacts with, and is regulated by, other genes in the total complex. Although a human gene may be isolated by molecular cloning, there is no guarantee that the gene, however normal in its sequence of nucleotides, will achieve proper regulation of expression when inserted in a new host environment. Nevertheless, the current pace of research is rapid, and the treatment of human disorders clinically by gene therapy is not too distant in the future.

The focus of gene therapy is on debilitating, relatively rare inherited disorders, primarily enzyme deficiencies caused by simple mutations. Even when the techniques become improved, only a few of the 3,000 known single-gene disorders are likely to be treatable. Indeed, the requisite biochemical basis is scarcely understood for most genetic disorders. Since the actual repair of a faulty human gene *in situ* is not possible with existing technology, the current strategy entails the insertion of a functional gene to offset the effects of the abnormal gene.

Modern molecular procedures have permitted the isolation and cloning of DNA sequences coding for specific gene products. It is now possible to cut complex genomic DNA molecules into fragments that can be ligated into a vector DNA molecule (such as lambda bacteriophage) and propagated perpetually in a host bacterium (Maniatis *et al.*, 1978). The ingenious method can generate numerous copies of human genes of medical interest. With the isolated gene on hand, the next step is to insert the gene inside the nucleus of the target cell. Several techniques have been devised, but attention of late has centered on defective RNA viruses (retroviruses) as the most reliable vehicle for transfer. Much of the native genetic information of retroviruses can be deleted, and replaced by exogenous DNA sequences.

In 1984, Richard C. Mulligan and his associates at the Massachusetts Institute of Technology "packaged" a retrovirus to transfer an antibiotic-resistant gene from a particular strain of bacteria to explanted bone marrow tissue of an irradiated mouse (Williams *et al.*, 1984). The targets for gene insertion were the hemopoietic stem cells, a self-sustaining population from which the blood cells are derived. These pluripotent stem cells comprise less than 0.1 percent of the marrow cells. If the stem cells were to incorporate the antibiotic-resistant gene introduced by the engineered viruses, the recipient mouse would possess the marker gene in every type of differentiated blood cell derived from the stem cells carrying the implanted gene. In conformity with expectation, the antibiotic-resistant gene was found to be present in differentiated white blood cells of the spleen. This finding was clear-cut evidence of successful gene transfer since the mature blood cells of the spleen of the irradiated host mouse could only have originated from the introduced hemopoietic stem cells. The efficiency of gene transfer was only 20 percent—that is, the recombinant viruses integrated only in a subpopulation of the stem cells. Moreover, the viruses could not be targeted to particular sites in the chromosomes. Nonetheless, these results, as well as others, are encouraging (Joyner *et al.*, 1983; Mann *et al.*, 1983; Miller *et al.*, 1984).

The immediate hopes for somatic cell therapy in humans revolve around those diseases that can be treated by manipulating hemopoietic cells of the bone marrow, since this tissue can be readily removed, manipulated *in vitro*, and easily reintroduced into an intact host. Several heritable immunodeficient disorders are manifested primarily in bone marrow-derived cells, prominent among them purine nucleoside phosphorylase (PNP) deficiency and adenosine deaminase (ADA) deficiency. Both rare disorders are conspicuous by their predisposition to recurrent and persistent infections. ADA deficiency attracted public notice because of David, the remarkable "bubble boy." David, whose last name has been withheld for privacy, died at age 12 in a Houston, Texas hospital on 22 February 1984. David spent all but his last 15 days in a germ-free plastic bubble.

In ADA deficiency, the failure to produce the appropriate enzyme in purine metabolism causes a marked impairment of both cell-mediated (T-cell) and humoral

(B-cell) immunity. Even the production of a small fraction of the normal enzyme activity (10–15% of normal) would be beneficial to the patient. The hope is that enzyme production can be fostered by placing the appropriate normal human gene via a virus into human patients. The protocol calls for removing the defective bone marrow from a patient, inserting a normal enzyme-producing gene into a number of marrow cells, and then reimplanting the treated bone marrow into the patient.

Although the techniques of somatic cell therapy have become increasingly refined, clinical trials in humans await additional studies with laboratory animals to obviate potential risks to the patient. There continue to exist great uncertainties about the eventual outcome of transferring a human gene (Anderson, 1984; Grobstein and Flower, 1984). So far, there is no assurance that the transferred gene will function normally or predictably. The means of programming the virus so that the human gene is inserted into a specific region of the chromosome have yet to be devised. The incorporation of the foreign gene in an inappropriate site is potentially dangerous. The improperly inserted gene may alter the function of neighboring genes, producing mutational alterations that can be detrimental to the host (Varmus *et al.*, 1981; King *et al.*, 1984). The virus itself may become unstable in its new site and become infectious or virulent. One grim consequence of improper insertion is the activation of cancer-producing genes, or *oncogenes*. There is the possibility that the transferred gene will become located near one of the 50 known oncogenes whose expression could lead to malignancy (Bishop, 1983; Croce and Klein, 1985).

Evidently, before clinical trials in humans are contemplated, it is necessary to weigh the potential risks to the patient, including the possibility of producing a pathologic virus or inducing a cancerous growth, against the anticipated benefits to be gained from the insertion of the functional gene. With crippling and life-threatening disorders, the adverse risks may be low compared to the severity of the disease that might be treatable by gene therapy. In essence, for the foreseeable future, somatic gene therapy is likely to be applied only in rare, specifically chosen genetic disorders under well-defined guidelines (Fed. Reg., 1985).

Monitoring human embryos

The technique of *in vitro* fertilization is no longer only of academic interest. The procedure has gone beyond the research stage and can now be considered as an established form of treatment for certain forms of infertility (Edwards and Purdy, 1982; Walters and Singer, 1982; Wood and Trounson, 1984; Seppala and Edwards, 1985). Presently, *in vitro* fertilization clinics have been established in about 28 countries. It is likely that *in vitro* fertilization programs will, in the not too distant future, be part of standard gynecologic services offered at major medical centers. Parenthood, even when it originates in the laboratory, is still an exalted event.

Chromosomal abnormalities do occur with high frequencies in human conceptuses that are produced by *in vitro* fertilization, as they do in embryos produced naturally. The chromosome constitution of the embryo can be established from cytological analyses of but a few cells. Roslyn Angell and her colleagues at the University of Edinburgh have described a method of examining chromosomes in 8-cell human embryos (Angell *et al.*, 1983). Accordingly, the possibility exists of determining the chromosome makeup of an 8-celled or 16-celled conceptus by removing one or two of its cells and culturing them for chromosomal analysis. The conceptus in the meantime would be frozen and afterwards thawed out and transferred to the woman only if the cells were shown to be normal. Lest one be concerned about the loss of one or two cells, it has already been shown that a frozen 8-celled mammalian conceptus that has lost some of its cells can give rise to a normal fetus (McLaren, 1982). As the molecular techniques become more sophisticated, the cell removed from the early embryo can be assessed not only for chromosomal aberrations, but for certain defective genes that cause debilitating malformations.

The technique of *in vitro* fertilization has made the embryo accessible to a degree of manipulation not previously possible (Grobstein, 1981). Monitoring for mutant genes and abnormal chromosome complements in the pre-implantation embryonic stage has the benefit of obviating anxiety stemming from diagnosis of the fetus *in utero* that involves invasive techniques such as amniocentesis. Prevention before implantation would circumvent the necessity of an abortion at a later stage of pregnancy. Even better, some thoughtful observers have argued, would be the replacement of the faulty gene with the normal gene so that the pre-implantation embryo can be saved. Once proficiency is gained in monitoring genetic abnormalities in the embryo, is it inevitable that we will opt to correct the genetic defects in the embryo?

Germ line therapy

Most individuals are comfortable with the idea of inserting genetic material in a human patient if the procedure is used for the sole purpose of compensating for malfunctioning genes and overcoming severe genetic defects. In other words, the remedial therapy affects only the treated patient and not later generations. In certain circles, anxieties have surfaced concerning the deliberate genetic modification of the gametes or embryos, such that the induced changes are passed on to the offspring (Fletcher, 1983; Friedman, 1983). The concerns are real in that the genetic transformation of certain traits in laboratory animals has already been accomplished by modern DNA technology (Palmiter and Brinster, 1985).

Several investigators have reported success in introducing foreign DNA into the germ line of experimental animals, notably mice, by microinjecting DNA into the pronuclei of fertilized eggs (Gordon *et al.*, 1980; Brinster *et al.*, 1981; Costantini and Lacy, 1981; Harbers *et al.*, 1981; Wagner *et al.*, 1981; Palmiter *et al.*, 1982). The foreign genes that have been injected include those that code for herpes virus thymidine kinase, human interferon, rabbit β-globin, growth hormone, mouse metallothionein, immunoglobulin, and rat elastase. In many instances the purified genes introduced into mice at the onset of embryogenesis are stably integrated and even expressed in the host genome. To cite one report among many, Thomas Wagner and his colleagues (1981) transferred by direct microinjection the gene coding for rabbit β-globin into the male pronuclei of mice zygotes. The resulting embryos were cultured *in vitro* to the blastocyst stage and then implanted in the uteri of pseudopregnant females. Not only did the microinjected rabbit gene become integrated into the genome of the mice that were born, but the rabbit gene was transmitted to the next generation of mice. Such laboratory mice in which foreign DNA has been stably integrated into the germ line have been called *transgenic* mice.

Perhaps the most dramatic of recent reports involves the correction, at least in part, of an hereditary growth deficiency by providing a dwarf mutant mouse with growth hormone by gene therapy (Hammer *et al.*, 1984). These investigators inserted a normal gene coding for a growth hormone (from a rat) into embryos of a mouse. The embryo evidently had integrated the transplanted growth gene—the newborn were almost double the size of their untreated brothers and sisters. In turn, many of the offspring of the treated individuals retained both the transferred gene and the uncharacteristic large size.

These pioneering experiments have not been free of troublesome complications. Only about 1 percent of the inoculated eggs developed into mice that expressed the microinjected gene. Moreover, the newly introduced gene failed to produce the proper amount of the desired product; the gigantism that resulted among the offspring signified that the implanted gene was producing unregulated excessive amounts of growth hormone. Finally, the foreign gene unsuspectedly had adversely affected other characteristics of the animal. In particular, the chronic elevated production of growth hormone had impaired the fertility of the females. All these unfavorable features argue persuasively against applying the present imprecise technology to humans.

The results are particularly disappointing in relation to correcting a defect that could be made transmissible to further generations. Since there is no way of integrating the foreign gene to a predetermined chromosome site, it is not possible to replace the mutant sequences with normal sequences. Accordingly, the inserted foreign gene will most likely segregate independently from the native mutant gene, with the outcome that both the correcting locus and the defective locus—and the potential for disease—will still be transmitted to the progeny. Evidence is also accumulating that the randomness of insertion bears a significant mitogenic potential (Schnieke *et al.*, 1983; Wagner *et al.*, 1983).

It has been said that the correction of a genetic disorder by gene therapy on an early embryo might be defensible if there were no alternative, more effective techniques available. Consider sickle-cell anemia, an enfeebling single-gene blood disease. This disease can be detected prenatally by analysis of fetal cells of the amniotic fluid (amniocentesis) or fetal cells of the placenta (chorionic villus sampling). Thus, prenatal diagnosis offers the parents the option of terminating the pregnancy if the fetus is demonstrably affected. But not all parents wish to avail themselves of prenatal diagnosis because they are unwilling to accept the option of abortion. Moreover, some genetic disorders, including sickle-cell anemia, may not be judged by the parents to be sufficiently incapacitating to warrant termination of pregnancy. For some parents, then, gene therapy on the gametes or embryo might be the only acceptable, if not efficient, means of preventing the transmission of specific malfunctioning genes to their immediate offspring, as well as future generations. The wish to eliminate the faulty gene once and for all from the family lineage might be very compelling.

PUBLIC REACTION

Few events are more illustrative of the rapidity and direction of change in our times than the impact of sophisticated developmental and reproductive technologies on human life. Each novel development strains our cultural and moral fabric. The ethics of gene therapy, *in vitro* fertilization, and other techniques are controversial. It has been said that the American public has largely remained silent on the profound ethical questions raised by the new reproductive and prenatal procedures. It has been suggested that our society has become morally bankrupt. On the contrary, it appears that the public at large has not grown indifferent but has become increasingly tolerant of individual differences.

A democratic, pluralistic society seems to be embracing the ideal of *respect for persons.* A central feature of this ideal is that persons are to be treated in ways that respect their freedom of choices. An individual has the freedom to apply knowledge gained from medical advances to achieve (or avoid) reproduction, while at the same time treating with impartiality those who would not themselves use such freedom. Persons who want to avail themselves of prenatal diagnosis are free to do so; those who do not are not blameworthy. In essence, each person exercises freedom of choice, and no person's rights are to be compromised in the exercise of a given choice.

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The Next Big Problem in Developmental Biology[1,2]

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The "next big problem" I want to describe here is deceptively simple. We all recognize that in the fertilized egg there is a set of genes (along with some cytoplasm, a matter which we shall consider later) and that these genes give instructions that ultimately produce a complex adult. Genes are known to produce messenger RNAs which are then translated into specific proteins in the cell cytoplasm. How is it possible that this very limited process can construct, for instance, a complex animal with different parts and different cell types all of which exist in harmony, and not only produces a consistent form from generation to generation, but also permits that form to move, to function in an organized way, and on top of all that, to behave? The phenotype, both in its structure and in its activities, seems remarkably far removed from those first gene products, yet they are its origin. So not only do genes make proteins, but the proteins can in turn do things that, through a chain of events, lead to the ultimate result. This is the problem: how do those genes and their proteins control such distant, remote events.

One way to study the matter is to look more closely at the properties and the activities of the genes, especially of ones that seem to play a crucial role in development. This has been and is being very actively pursued in many laboratories with enormous success. It is the exciting frontier that is moving forward with dazzling speed. However, the steps that follow are equally important and exciting and they are the ones that have been more recalcitrant in revealing themselves, although we understand a large amount. But, as D'Arcy Thompson said, "Nature keeps some of her secrets longer than others."

I plan to discuss "the problem" here by examining how it arose in the first place: what is its evolutionary origin. This will bring me to the rather basic question of why we have development at all, and at the same time show that in an interesting way the rise of development is related to the rise of complexity. Within this setting I shall then try to organize some of the major concepts of development to show how they bear on the problem of how genes can have effects so remote from their immediate products. This will end with a brief discussion of behavior, the ultimate as a process that distances itself from the genome, and the ultimate in complexity.

THE EVOLUTION OF SIZE INCREASE

If one looks at the fossil record from the earliest beginnings and then follows it over billions of years, a striking fact emerges. The largest forms three and a half billion years ago were single cell bacteria, but with time the maximum size records steadily increased, first with larger cells and simple multicellularity, and then with the appearance of eukaryotic cells multicellular forms kept pushing the size limit upwards through geological time until finally we have the blue whales and giant sequoias of today. This progression does not mean that the smallest or middle-sized organisms have disappeared, for obviously they have not. It simply means that the upper size limits for many different groups of animals and plants has slowly increased over time.

When we ask why this might be so, it is possible to argue that there is often selection pressure for size change, or in some instances the change may be due to drift, especially in small populations. There are

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[1] From the Symposium on *Science as a Way of Knowing—Developmental Biology* presented at the Annual Meeting of the American Society of Zoologists, 27-30 December 1986, at Nashville, Tennessee.

[2] This essay is an abstract of a portion of a forthcoming book, *The Evolution of Complexity*, in which detailed references are given.

many studies, both geological and ecological, which support the idea that size changes have occurred over time, but those changes may be either for an increase or a decrease in size. If size changes can result from selection, one escape from competition and predation is to become larger than any other organism. This is a reasonable explanation for the ever expanding upper size limits during the course of evolution.

COMPLEXITY AND SIZE

One of the automatic consequences of size increase is an increase in complexity. Complexity means the increase in parts and the interrelation of those parts. In living organisms the concept of complexity can be identified as differentiation, a form of division of labor.

If one is to argue that size increase is correlated with a division of labor within organisms one needs some way to gauge the accuracy of this generalization quantitatively. It is easy to measure size but difficult to measure complexity. One could say that since cells differentiate into cell types with different functions, then one might simply count the number of cell types in any animal or plant and this would be an index of complexity. The difficulty is that it is very hard to agree on how many cell types exist in large organisms such as trees, or ourselves. Nevertheless, the idea is useful even if one's estimates are considerably inaccurate. The reason for this is that one can make some general categories which have approximately a certain number of cell types, and then put all the appropriate groups of organisms in that basket. For instance, higher plants might be considered to have in the neighborhood of roughly 30 cell types, while vertebrates will have, by any system one uses for identification, over 120 cell types. This means that one can say with some confidence that vertebrates have more cell types than angiosperms, even though the exact numbers are to a considerable degree uncertain.

After examining the maximum and minimum size for different groups of organisms with numbers of cell types from 1 to over 120, it can be seen that there is clearly a trend despite the fact that for any one group the range of sizes might be enormous. Consider for instance that an angiosperm can be a minute duckweed or a huge eucalyptus tree, and a vertebrate will range from a minuscule fish to a giant whale. Yet despite the extremes of these minima and maxima for each group, there is clearly a trend: larger organisms are more complex. That this should be so can be explained on mechanical principles. A large organism simply could not function unless it had an appropriate division of labor which makes it possible to function. Size affects how an organism takes in food and oxygen, how it gets the essential substances to all its metabolizing cells, and how it eliminates its waste products. These and many other aspects of the running living machine are all size related; there could be no other way.

WHY DEVELOPMENT?

One invariant property of organisms is that the larger they are, the longer and more complicated their development. The reason for this is that virtually all organisms have a single cell stage in their life cycle. In some forms it may be a spore, but far more generally it is a fertilized egg. The reason that this is so has to do with the ubiquity of sexuality and the replication and recombination of the genetic material. In eukaryotes it occurs in chromosomes which are capable of mitosis and meiosis. This wonderfully clever system of duplicating and reshuffling the DNA for a new generation, a new life cycle, is so successful that once invented it has remained virtually unchanged (at least in any major way) during the evolution of all organisms from the first eukaryotes. Perhaps the most important point is that the uniting of two genomes by fertilization, the key element in sexuality, is possible only in single cells where two haploid gametes fuse to form a diploid zygote.

If, then, we have one stage of the life cycle that is large and multicellular (the adult) and another that, for reasons of properly controlling the genetic material, must be a single cell stage, the inevitable result is development. To put it in a very general way, development is the direct consequence of the evolutionary success of sex and size.

THE ACTIONS OF GENES

How genes behave and how they do things, as I said earlier, is an exceedingly active field at the moment, and here I want only to discuss a few small points that may give some insights into the power of developmental genetics.

First let me point out that genes can, by very small changes in their sequence of codes, make very radical changes in the structure of the proteins that they produce. This is because proteins have a tertiary structure which is contingent on certain amino acids on the protein chain reacting or associating with others, and depending where those key, mutually reactive amino acids are located, the folded, tertiary structure will be greatly affected. Suppose the protein is an enzyme and its activity depends upon its tertiary structure, then one small change in a nucleotide in the DNA will affect the ability of the enzyme to catalyze a reaction; it may decrease or eliminate that power or it may enhance it. But already the effect of the DNA is considerably removed from the end result. The enzyme may be allosteric, that is, can be affected by chemical combinations with other substances, including its substrate, which will either increase or decrease its reactivity (positive or negative cooperativity). In other words the tertiary structure has taken on a life of its own and can gain new properties by slight changes in its configuration due to the substances which surround it and combine with it. Those allosteric changes are not coded in the DNA; all that is coded is a chain of amino acids which produce a complex tertiary structure that is responsive to the chemical changes in its environment. This means that already at the level of proteins there are activities that arise which are not directly controlled by genes. The genes merely set the stage and provide the capacity to have those activities. In this simple example we already see how genes may produce remote effects beyond their immediate supervision.

To this simple picture, let us now add the fact that not all the proteins produced by genes are enzymes. Some genes produce regulatory proteins whose only role is to affect the action of other genes so that there is a network of cross reactions providing a hierarchy of gene functions. Such regulatory genes are known to play a major role in controlling key events during development; they are master switches.

Another level of complexity is seen in the phenomenon of pleiotropy. Here one gene may have numerous effects on the phenotype. A gene might affect eye color in *Drosophila*, but also produce changes in the gut. The effects of pleiotropic genes are often multiple. It is presumed in these cases that either the protein which is the direct gene product, or one of the substances derived from that protein through subsequent chemical steps, has quite specific effects in different parts of the body that do not seem in any obvious way related. Pleiotropy must be thought of as an indication of the hidden complexity of the action of genes and their subsequent effects through intertwining chemical pathways. This raises the vision of development as being a maze of interconnected chemical sequences of such dark complexity that one might wonder how we could ever unravel them.

SIGNAL-RESPONSE SYSTEMS

One way of stating the problem helps. Think of the developing organism as made up of a set of signals and a set of specific set of receptors for those signals. The genes are responsible either directly (if they are proteins) or indirectly (if they are small molecules) in producing both the signal substances and the receptors. In this way a communication system was set up within the embryo whereby events can be initiated or terminated or modulated. By making the receptor specific (and in some instances also the signal substance) it is possible to gain enormous powers of discrimination on where, in development, activation or inhibition will occur.

TIMING AND LOCALIZATION

One of the great problems in developmental biology is the timing of these signal-response systems so that they occur at the right moment, and furthermore that they are positioned in the right place. There are many interesting studies being made on the matter of timing mechanisms and hopefully soon we will have a better understanding of how they are controlled and how they relate to the genome.

The question of localization, or pattern formation as it is often called, is of enormous current interest. It is being attacked on two fronts. One is again by the developmental geneticist who is looking for the gene control of major patterns in the early embryos of nematodes, and especially *Drosophila*. There are genes which affect the major axes of polarity of the embryo and genes which affect segmentation. How those genes achieve these patterns is one of the genuinely exciting frontiers of developmental biology. But because so many aspects of localization, as was stressed earlier, are so far removed from the immediate actions of genes, this approach will not lead to the solution of all the problems of localization.

One of these approaches has been by mathematicians. The use of mathematical models in developmental biology is now a thriving industry that began some time ago. In recent years it has illuminated how it is possible, by means of reaction-diffusion models, or models involving mechanical parameters, to produce an enormous variety of pattern. This does not mean that the models can ever tell us what is actually going on inside the embryo—this can only be demonstrated by experimental dissection—but it can show us what is possible theoretically and, therefore, what sorts of things the experimentalist should look for. I think the importance of mathematics in developmental biology today and in the future is in danger of being underestimated. Those of us who work with molecules, with chemical reactions, with the cell biology of development are necessarily looking at the problem from a very narrow view. We are looking for solutions to simple questions; we have hardly any choice. But the mathematicians have already shown us that some of these little questions are in fact big ones, and we should pay attention to their models.

INTERTWINING CHEMICAL PATHWAYS

I would like to return to the idea that one chemical event in a cell leads to another, and these events may consist of long chains of chemical reactions that may branch and criss-cross in all sorts of intricate ways. Furthermore, they are affected by each other so that a product may inhibit (or stimulate) an early reaction in this way producing feedback loops (or there may be autocatalytic, feedforward loops). Presumably it is because of this kind of network that one has pleiotropic effects. One gene change might produce a variety of effects at the end of different but interconnected chains. Now let us consider such intertwining chemical pathways in different-sized animals and plants.

In small single cell organisms such as bacteria having all, or many chemical reactions connected does not raise too great a problem simply because the small size limits the number of steps and the number of connections. If a new mutation has diverse (pleiotropic) effects and one of them is deleterious, the result will be the death of the cell. But because bacteria can increase from a few cells and in a short time produce vast quantities of descendents, any lethal mutation will be cast aside, and any neutral or selectively advantageous mutation will increase at a rapid rate. These gene effects in bacteria are to some extent all-or-none and their rapid rate of turnover will multiply the good genes.

If we compare this situation with that of a large multicellular organism, we see that there must be some way to prevent excessive pleiotropy, excessive interconnections of all the gene initiated chemical pathways, for otherwise any chance of a successful mutation would be minute simply because even the smallest gene changes would have, if there were not some mechanism to prevent it, vast possibilities for destroying some essential process of the development or the function of the adult. Before discussing the buffering mechanism, let me give an example.

Breaking down cellulose into its component glucose molecules is a difficult task. This is not because cellulase is a difficult molecule for an evolving organism to invent, but because the cellulose is glued together in fibrils and a battery of enzymes is needed to separate the cellulose so that the cellulase can reach the cellulose. Only a very few invertebrates are known to have acquired the machinery needed to break down cellulose and no vertebrate, yet many animals, from termites to cows, eat cellulose. On the other hand, many unicellular microbes have independently invented the machinery: numerous bacteria, fungi, and protozoa. So the first conclusion is that it has been easier for the smallest organism to do this than large ones, for they have devised the magical enzymatic combination many times during the course of evolution, but among all animals it has been done only twice. The second conclusion is that for a large, complex animal it clearly must have been easier to take cellulose destroying microbes into its gut, and provide a favorable environment for these symbiotic slaves than going to the trouble (and making the extremely difficult evolutionary step) of making all the new enzymes itself.

Let us now return to the question of how large organisms with long and complicated developments manage to have genetic changes that run less risk of being lethal. To explain this I shall use a novel concept which I will call *gene nets.* The idea is straightforward. The activities of genes are grouped and these groups of genes and their immediate products and their subsequent chains of reactions are not all interconnected throughout the multicellular organism, but they are isolated into groups which are the gene nets. This means, for instance, that one might have a gene which produces a specific defect or change in one organ, such as white eyes in *Drosophila*, but because that gene is isolated in a gene net that is associated with the development of the eye, it has no other effects. (Presumably not all genes would be in gene nets, for those associated with, for instance, the basic metabolic machinery would apply to all the cells of the body. Gene changes relating to such basic metabolic process could indeed be pleiotropic, such as the eye color-gut defect mentioned earlier. But successful metabolic mutations are rare because they are not protected and are vulnerable. This is one area of the genome where one sees little change during the course of evolution of major groups of organisms.)

It is by the formation of gene nets in complex organisms that heterochrony is possible. In a classic example, in salamanders in general the larval stage is aquatic and the larva has gills, while the adult has lungs. In the Mexican *Axolotl* the gonads ripen in the larvae which may never reach adulthood, always remaining aquatic. Here we have two gene nets: one for the production of lungs and one for the ripening of the gonads. The timing of these two gene nets can be shifted so that the order in which they appear can be reversed. This is heterochrony, and such a shift would not be possible if both structures were interconnected on the same set of chemical reaction pathways. So gene nets make it possible not only for genetic mutation to affect different parts of a large organism without the danger of having adverse effects on other parts, but also to shift the timing of developmental events, often producing major evolutionary steps.

EXTREME DISTANCING OF THE FINAL DEVELOPMENTAL RESULT FROM THE INITIAL GENE ACTION

Thus far the principles of how the phenotype may be far removed from the initial gene product have been examined. Next I want to discuss two examples in which that distance seems remarkably remote. The first is the particularly interesting case of the social insects, and the second is behavior.

Social insects

In the case of social ants, bees, wasps, and termites there is not just a division of labor within any one individual, but between individuals as well. Besides the male and the queen there is a large family of neuter workers which, in one of the more complex social insects, may have a number of different sizes and size-related shapes. Furthermore, their activities within the colony represent a clear division of labor. The smallest workers help the queen and take care of the larvae in the nest. The middle-sized workers usually specialize in food gathering and storing. The largest workers are the soldiers that are specialized for guard duty and keep out unwanted predators.

Not only does this division of labor exist, but like the division within an individual organism, the parts (individuals) are genetically closely related, and clearly the difference arises from external influences. For instance, in the case of termites the soldiers are known to give off an inhibitor that prevents the young nymphs from molting into soldiers. If all the soldiers are removed from a colony, the inhibitor they give off disappears and immediately new soldiers appear at the next molt. By means of such inhibitors a balance between the castes is achieved and we have what might be considered pattern formation (*i.e.*, controlled proportions) at the level of the colony. This is, in essence, a developmental stimulus-response mechanism between individuals rather than within individuals. The inhibitors between individuals affect the endocrine balance within the nymphs and in this way the direction of their differentiation is guided. If we now turn this picture around and ask how the genes control the proportions of the castes, one can see that there is a considerable hierarchy of events. The signal-response systems act not only at the level of the cells, but at the level of the organs within the individual insects, and then ultimately at the level of the colony, that is, between insects. There is a long path from the first proteins made by the genes to the integration of a colony of social insects.

Developmental plasticity and the formation of the brain

It is evident from the example of social insects that the development of the workers is to some degree plastic and the direction in which they are pushed depends upon external factors such as inhibitor substances and other external factors which I did not discuss such as nutrition. There are many examples where a particular developmental pathway may be influenced by the environment. For instance, the crowfoot, an aquatic buttercup, will develop entirely different-shaped leaves depending upon whether they are under water or in the air.

Another kind of plastic development may be seen in the construction of the vertebrate brain and nervous system which is of particular interest. It has been well established that the exact number of neurons needed in the brain is not fixed. Instead a considerable excess of neurons is made and only after they have moved about in the early forming brain and found connections does the number become fixed. All those that did not make it into the organized, connected network of nervous tissue die. This means that potentially there could be considerable variation in the number of neurons in two brains of closely related animals. We do not understand what are the control mechanisms for connecting of the young nerve cells. One could imagine that a combination of chance movement and early function might play an important role, but still this must be thought of in a background of genetic information. It will be very important to understand this process in greater detail.

Behavior

As I said earlier, development is the ultimate complex process that is far removed from the first gene transcriptions and translations. Furthermore, it is the ultimate in complexity, and the ultimate in plasticity. We need only think of our own thoughts to be convinced that is the case. It is so evident that we may even question that genes have anything to do with our behavior, but only provide the neuronal setting from which behavior can emerge. It is much easier to obtain some picture of the situation if we briefly examine the behavior of some non-human animals.

Consider the case of predator avoidance in birds. As we know from some of the early experiments of the pioneers in ethology, if a hawk-like silhouette is pulled on a wire over young goslings they will scuttle for cover, even though they may be only a few hours old and have never seen a hawk before. The shape is quite specific, for reversing the direction of the silhouette (which makes it look like a goose) does not cause the alarm reaction. From this we must conclude that this is a complicated reaction which involves recognizing a particular visual pattern and hooking it up with a specific response pattern. Since it is ready to operate at birth we assume that it has a genetic basis.

It is not at all clear how the genes specify such an involved bit of behavior. One can only assume that the process is very complex, involving many chemical steps, building up a signal-response system which involves widely different and separated parts of the body. This clearly has occurred during development, and therefore it is a particularly fascinating problem in developmental biology, one we hope will receive careful attention in the years to come.

Let us now expand on this theme with examples of ever increasing complexity and plasticity. Another form of predator avoidance in birds is seen in mobbing. A flock of crows will, for instance, surround a large owl and make a tremendous commotion cawing and lunging at the owl. It is an assumed that this behavior helps to draw attention to the source of danger so that no crow is caught unawares, and it also is often successful in making the predator move off to another territory. There is considerable evidence that at least some of this behavior is learned. In a set of elegant experiments, E. Curio and his colleagues (1978) set up two cages so that one European blackbird saw an owl and the other a harmless Australian honeyeater. The first bird began to energetically mob the owl, and the other bird witnessed all of this frenzy. Before long it began to mob the honeyeater (which it never had done in a control experiment). The bird that had been taught the presumed dangers of the honeyeater was, in the next experiment, presented with that same harmless bird which it proceeded to mob, and a new, naive bird, which could also see the honeyeater, soon followed suit and mobbed it too. This bit of cultural information was passed on in five other passages from a new learner to a new naive bird. In this case we assume that the advantage of such learning to a bird is that if new predators enter the territory it will not take thousands of generations to learn the new danger; it can be passed on in a matter of minutes. The flexibility which learning imparts to behavior could conceivably be of enormous adaptive advantage to an organism to cope with rapid changes in its environment.

One final example where there is even greater flexibility in behavioral signal-response system may be seen in African vervet monkeys which have been extensively studied by R. M. Seyfarth and D. L. Cheney (1984). They show that these monkeys have three vocal signals, each one for a different danger. One, which is the signal for an eagle, causes all the members of the social group to scurry for protection in clumps of bushes. Another distinct alarm call means leopard, and to this they rush out into the most open spot where they are least likely to be ambushed. Finally, the third call is for snakes, also a dangerous predator (in this case usually a boa constrictor). Upon hearing this signal the monkeys quickly look around the ground, and then scamper up the nearest tree. It is clear that to one situation, predator avoidance, the vervet monkeys have produced a variety of signals to accommodate their multiple needs. We do not know how much is inherited and how much is learned, although there must be a large component of the latter. The main role of the genes is to set down an anatomical structure, the nervous system including the brain, which is capable of such a variety of flexible responses.

CONCLUSION

All behaviors, including the ones described above, are the product of genes. Even their amazing plasticity is possible only because of the genes and the proteins they make. What the genes have produced over the course of evolution of larger and more complex organisms seems to have become increasingly remote from those initial gene products. The remoteness lies entirely in the number and complexity of the intervening steps. Let me briefly review the generalizations I have made about those steps.

Following the first proteins that are translated, new properties may emerge, even in the proteins themselves, that can be explained by the sequence of the amino acids. Those properties include changes in tertiary structure which lead to the flexibility of allostery. These proteins as enzymes, or substrates, ultimately control a whole series of interlocking, sequential chemical reactions that produces the obvious morphological changes we see during development. Those changes become self regulatory by means of feedback and feedforward loops, and they often produce ultimately a complex of signal-response systems which also are key regulatory elements during development. The signals and responses can occur at different levels, cells, multicellular individuals, and even in social groups. Moreover, the signals can go from one level to another, in this way binding the levels by a system of intercommunication. With increase in size these communication systems have become grouped into what I have called gene nets, and each gene net has achieved some degree of autonomy so that it can accommodate internal changes without necessarily affecting the workings of other gene nets in the developing organism. Such postulated partitioning is a way to handle increased complexity.

The zenith of developmental complexity is the evolution of the nervous system and the brain. It gives rise to behavior which appears to be a very different kind of complexity from anatomical intricacies. Furthermore, its cellular development and the development of its activity, especially in the interplay of rigid, determined activity with highly flexible activity, seen especially in learning, seem to be two totally separate mechanisms that complement each other. Behavior is a new kind of development, for indeed it is a new kind of invention of the genome, and for that reason alone it is intimately part of development.

In the great span from bacteria to the monster plants and animals of today, there has been an increasingly elaborate matrix of steps between the genes and the end result. That is the great problem of developmental biology.

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Films and Videotapes in Developmental Biology[1]

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Numerous 16 mm films and videotapes are available to supplement teaching of the topic, developmental biology. However, during our survey of various resources, we learned that titles for this topic are normally indexed under the headings of embryology, reproduction, cell biology or laboratory techniques, rather than that of developmental biology per se. Below, we have listed a number of titles which, based on film catalogue descriptions, we feel would probably prove useful in classroom and/or laboratory teaching of developmental biology. Since a number of them are rather brief and specialized, we have included running times in parentheses. Most of the films and videotapes listed are available for rental and/or purchase through major film libraries and distributors. A good way to obtain additional information regarding films in biology is to contact a media specialist in either your own institution's film resource center or one of the major film centers listed in Appendix I. Resources we have found useful in our work include the *Index to 16 mm Educational Films* (8th ed., 1984) and *Index to Educational Videotapes* (6th ed., 1985). Both of these indices are published by NICEM (University of Southern California, University Park, Los Angeles, California 90007). Another excellent resource is the latest edition (1986) of the *Educational Film/Video Locator of the Consortium of University Film Centers and R. R. Bowker Co.* (R. R. Bowker Co., New York, New York). The latter two volume publication is particularly informative because it provides not only titles, descriptions, available sources, and subject listings of films and videotapes but also detailed information regarding lending policies of the member institutions of the Consortium of University Film Centers. For your convenience, we have listed these institutions in Appendix I.

Films and videotapes which could be utilized for the topics evolutionary biology, human ecology, and genetics may be found in the proceedings of previous *Science as a Way of Knowing* symposia (Hart, 1984; Anderson and Hart, 1985; Hart and Anderson, 1986).

LIST OF FILMS

Aggregation of Dissociated Sponge Cells (8)
Amphibian Embryo (16)
Animal Reproduction: The Wonderful Process of Creating a New Life (17)
Ascidian Metamorphosis: The Role of Contractile Epidermis in Tail Resorption (10)
Auto Inhibition in a Fungus (6)
The Baby Makers (42)
The Beginning of Life (30)
Beginning of Life: The Mammalian Story (26)
Beginnings of Vertebrate Life (11)
Behavior of the Endoplasmic Reticulum in Living Cultured Cells (10)
Beyond the Naked Eye (18)
Biography of the Unborn (15)
Birth of the Red Kangaroo (21)
Blueprint for Survival (20)
Cell Differentiation: The Search for the Organizer (15)
Chick Embryo Explantation (8)
The Chick Embryo: From Primitive Streak to Hatching (13)

Chick Embryo: Life is Born (15)
Chick Embryology (11)
Chick Embryo Techniques (15)
Chicken or Egg? (23)
A Clone of Frogs (15)
Colony Formation in *Pediastrum* (7)
Contact Inhibition of Movement in Fibroblasts (4)
Cytoplasmic Motility (5)
The Day Life Begins (23)
Deficiencies in the Decorticate Pigeon (3)
The Determination of Egg Polarity by the Environment (6)
Development and Differentiation (20)
Development and Metamorphosis of the Leopard Frog: *Rana pipiens* (25)
Development in *Volvox* (15)
Development of an Egg (10)
Development of a Salamander (10)
Development of Behavior in the Duck Embryo (21)
Development of Cellular Slime Molds (9)
Development of *Drosophila melanogaster* (17)
Development of Organs (29)
Development of the Axolotl (8)
Development of the Cardiovascular System of the Chick: The Blood Vessels (23)
Development of the Cardiovascular System of the Chick: The Heart (20)
Development of the Chick (11)
Development of the Chick Embryo (6)
Development of the Chick Embryo (16)
Development of the Chick: Extraembryonic Membranes (20)
Development of the Newt Germ (15)
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Boston University
Krasker Memorial Film Library
565 Commonwealth Avenue
Boston, MA 02215
617/353-3272

Brigham Young University
Audio Visual Services
101 Harvey Fletcher Building
Provo, UT 84602
801/378-2713

Central Washington University
Media Library Services
IMC
Ellensburg, WA 98926
509/963-2861

Cornell University
Audio Visual Center
8 Research Park
Ithaca, NY 14850
607/255-2091

Eastern New Mexico University
Film Library
Portales, NM 88130
505/562-2622

Florida State University
Instructional Support Center Film Library
54 Johnston Bldg.
Tallahassee, FL 32306-1019
904/644-2820

Idaho State University
Audio Visual Services
Campus Box 8064
Pocatello, ID 83209
208/236-3212

Indiana State University
Audio Visual Center
Stalker Hall
Terre Haute, IN 47807
812/237-2690

Indiana University
Audio Visual Center
Bloomington, IN 47405
812/335-2103
800/552-8620
800/942-0481 (IN)

Iowa State University
Media Resource Center
121 Pearson Hall
Ames, IA 50011
515/294-1540

Kent State University
Audio Visual Services
330 Library Bldg.
Kent, OH 44242
216/672-3456

Louisiana State University
16mm Film Library
118 Himes Hall
Instructional Resource Center
Baton Rouge, LA 70737
504/388-1135

Michigan State University
Instructional Media Center
East Lansing, MI 48824-0610
517/353-3960

North Texas State University
Media Library
P.O. Box 12898
Denton, TX 76203-2898
817/565-2691

Northern Illinois University
Film Library
Altgeld 114
Dekalb, IL 60115
815/753-0171

Oklahoma State University
A-V Center
Stillwater, OK 74078-0383
405/624-7216

The Pennsylvania State University
Audio Visual Services
Special Services Bldg.
University Park, PA 16802
814/865-6314
800/826-0132
(outside PA)

Portland State University
Film Library
Portland, OR 97270
503/229-4890

Purdue University
Film Library
Stewart Center
West Lafayette, IN 47907
317/494-6742

Southern Illinois University
Learning Resources Services
Carbondale, IL 62901
618/453-2258

State University College at Buffalo
Film Library Communication Center 102
1300 Elmwood Avenue
Buffalo, NY 14222
716/878-6682/6821

State University of New York at Buffalo
Media Library
24 Capen Hall
Buffalo, NY 14260
716/636-2802

Syracuse University
Film Rental Center
1455 E. Colvin St.
Syracuse, NY 13210
315/479-6631

University of Arizona
Film Library
Audio Visual Bldg.
Tucson, AZ 85721
602/621-3282

University of California
Extension Media Center
2176 Shattuck
Berkeley, CA 94704
415/642-0460

University of California/Los Angeles
Instruction Media Library
Powell Library, Room 46
Los Angeles, CA 90024
213/825-0755

University of Colorado
Academic Services
Box 379
Boulder, CO 80309
303/492-7341

University of Connecticut
Center for Inst. Media & Technology
Storrs, CT 06268
203/486-2530

University of Idaho
Media Center
Moscow, ID 83843
208/885-6411

University of Illinois
Film Center
1325 South Oak St.
Champaign, IL 61820
217/333-1360
800/367-3456
800/252-1357 (IL)

University of Iowa
Audio-Visual Center
C-5 Seashore Hall
Iowa City, IA 52242
319/353-5885

University of Kansas
Film Rental Library
Continuing Education Bldg.
Lawrence, KS 66045
913/864-3352

University of Maine
Instructional Systems Center
12 Shibles Hall
Orono, ME 04469
207/581-2510

University of Michigan
Michigan Media
400 Fourth St.
Ann Arbor, MI 48103-4816
313/764-5360

University of Minnesota
University Film & Video
1313 Fifth Street, SE
Minneapolis, MN 55414
612/373-3810
800/847-8251
800/542-0013 (MN)

University of Missouri-Columbia
Academic Support Center
505 E. Stewart Rd.
Columbia, MO 65211
314/882-3601

University of Montana
Instructional Materials Service
Missoula, MT 59812
406/243-5976

University of Nebraska/Lincoln
Instructional Media Center
Nebraska Hall 421
Lincoln, NE 68588
402/472-1900

University of Nevada/Reno
Film Library
Getchell Library
Reno, NV 89557
702/784-6037

University of New Hampshire
Dept. of Media Services
Dimond Library
Durham, NH 03824
603/862-2240

University of Pittsburgh
Media Services
G-20 Hillman Library
Pittsburgh, PA 15260
412/624-4463

University of South Florida
Film Library
4202 Fowler Avenue
Tampa, FL 33620
813/974-2874

University of Texas at Austin
General Libraries
Film Library
Box W
Austin, TX 78713-7448
512/471-3572

University of Texas at Dallas
Media Services
P.O. Box 643
Richardson, TX 75083
214/690-2949

University of Utah
Instructional Services
207 Milton Bennion Hall
Salt Lake City, UT 84112
801/581-3170

University of Washington/Seattle
Instructional Media Services
23 Kane Hall DG-10
Seattle, WA 98195
206/543-9909

University of Wisconsin/La Crosse
Film Rental Library
127 Wing Communications Center
1705 State St.
La Crosse, WI 54601
608/785-8041
800/831-9504
800/362-8323 (WI)

University of Wisconsin/Madison
Bureau of Audio-Visual Instruction
1327 University Avenue
P.O. Box 2093
Madison, WI 53701-2093
608/262-3902
800/362-6888 (WI)

University of Wyoming
Audio Visual Services
Box 3273 University Station
Room 14 Knight Hall
Laramie, WY 82071
307/766-3184

Utah State University
Audio Visual Services
Logan, UT 84322-3100
801/750-2658

Washington State University
Instructional Media Services
Pullman, WA 99164
509/335-5618

Wayne State University
Media Services
5265 Cass Avenue
Detroit, MI 48202
313/577-1980

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Hart, N. H. 1984. Films, filmstrips and videotapes in evolutionary biology. Amer. Zool. 24:465–466.

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